Utilisation du comportement natatoire de Daphnia magna comme indicateur sensible et précoce de toxicité pour l'évaluation de la qualité de l'eau

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THÈSE EN CODIRECTION PRÉSENTÉE POUR OBTENIR LE GRADE DE

DOCTEUR DE

L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE SCIENCES & ENVIRONNEMENTS

LABORATOIRE NATIONAL HYDRAULIQUE ET ENVIRONNEMENT

SPÉCIALITÉ: Géochimie et Écotoxicologie

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Soutenue le 30 octobre 2014

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Utilisation du comportement natatoire de *Daphnia magna* comme indicateur sensible et précoce de toxicité pour l'évaluation de la qualité de l'eau

Résumé : Les paramètres comportementaux sont de plus en plus considérés comme étant des indicateurs sensibles et précoces de toxicité chez les organismes aquatiques. Cependant, l'utilisation du comportement natatoire comme critère de toxicité pour l'analyse de risque environnemental ou pour le contrôle de la qualité de l'eau reste pour l'heure encore limitée. En effet, il est actuellement difficile de quantifier la sensibilité des paramètres comportementaux et d'établir un lien entre les seuils d'effets comportementaux et les effets aigus et subaigus classiquement mesurés dans les tests en écotoxicologie. Dans le but d'améliorer notre compréhension des effets comportementaux des polluants sur *Daphnia magna*, nous avons développé un nouveau système de mesure du comportement multi-cuves baptisé « Multi-DaphTrack ». Douze substances toxiques, couvrant une large gamme de modes d'action différents ont été testées dans ce système. Un test standard d'immobilisation ainsi que des analyses de comportement dans le toximètre DaphToxI®, ont également été effectués pour chaque substance afin de comparer les seuils d'effets comportementaux avec les paramètres classiques d'immobilisation. Les résultats des expositions aux différentes substances ont démontré que notre nouveau système d'exposition multi-cuves permet de détecter des effets comportementaux (i.e., augmentation de la vitesse de nage) significatifs et précoces pour l'ensemble des substances testées et ce, à des concentrations proches de la CE_{10} (48 h) du test aigu d'immobilisation dès la première heure d'exposition. Des profils comportementaux différents ont été observés selon les substances testées (i.e., intensité, temps de latence et durée de l'effet) mais ceux-ci ne sont pas spécifiques d'un mode d'action particulier. Les résultats obtenus avec le toximètre DaphToxI® ont révélé des profils d'effet similaires (i.e., augmentation de la vitesse de nage) bien que ce système soit globalement moins sensible par rapport au système « Multi-DaphTrack ». Pour conclure, notre nouveau système d'exposition multi-cuves « Multi-DaphTrack » est un outil plus sensible et précoce que le test standard d'immobilisation pour l'évaluation de la toxicité de substances chimiques. L'utilisation du système « Multi-DaphTrack » pourrait donc être envisagée, après quelques améliorations et validation supplémentaires, pour l'évaluation de la qualité des masses d'eau et des effluents.

Mots clés : Comportement natatoire, indicateur de toxicité, *Daphnia magna*, biosurveillance, polluants, mode d'action

Use of *Daphnia magna* swimming behavior as a sensitive and early indicator of toxicity for water quality assessment

Abstract: Swimming behavior is increasingly reported as a sensitive and early indicator of toxicant stress in aquatic organisms. However, it remains unclear how to quantify the sensitivity of swimming behavioral endpoints and how to compare these effect thresholds with standard ecotoxicological endpoints used in risk assessment. To date, the systematic assessment of the sensitivity of swimming behavioral endpoints in daphnia is limited because of the restrained test capacity of existing behavioral analysis systems. Hence, we developed a new behavioral analysis multi-cell system named "Multi-DaphTrack" with a high throughput testing capacity in order to enhance our understanding of swimming behavioral effects in *Daphnia magna* under chemical exposure. Twelve compounds covering different modes of toxic action were selected and tested in this new system and in a single-cell commercialized biomonitor (DaphToxI®) and with the acute standard test. Our new multi-cell exposure system detected significant and early swimming behavioral effects (i.e., increase of the average speed) for most of the tested compounds and this, from the first hour of exposure at concentrations near the EC_{10} (48 h). Contrasted behavioral profiles were observed for average speed (i.e., intensity, time of effect onset, effect duration), but no distinct behavioral profiles could be drawn from the chemical mode of action. Despite less sensitive, the DaphToxI® gave similar trends (i.e., rapid peak increase) compared to our "Multi-DaphTrack" system. To conclude, behavior analysis using our "Multi-DaphTrack" system could be used as an alternative or complement to the current acute standard test for toxicity assessment of chemicals. With some additional improvements and validations, it also could be used for quality assessment of water bodies and sewages.

Keywords: Swimming behavior, toxicity indicator, *Daphnia magna*, biomonitoring, pollutants, mode of action

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Utilisation du comportement natatoire de *Daphnia magna* comme indicateur sensible et précoce de toxicité pour l'évaluation de la qualité de l'eau

RÉSUMÉ : Le développement exponentiel des activités industrielles, agricoles et urbaines engendre le déversement croissant de nombreuses substances dans l'environnement. Le compartiment aquatique est généralement le réceptacle final d'un bon nombre de ces composés qui menacent le fonctionnement et la pérennité de l'écosystème. Cette pollution diffuse, dû à la présence de contaminants de nature variée a entrainé une détérioration progressive de la qualité des eaux européennes menaçant ainsi la biodiversité et l'équilibre de l'écosystème aquatique. Une étude extensive recensant jusqu'à 4000 sites de biosurveillance sur l'ensemble du continent Européen a récemment révélé que la moitié des eaux de surface européennes était touchée par une pollution de contaminants organiques (Malaj et al., 2014).

Face à ces problèmes environnementaux, la Directive-Cadre Européenne sur l'Eau (2000/60/CE) a été adoptée le 23 octobre 2000 par l'Union Européenne avec pour objectif général l'atteinte du bon état écologique et chimique des eaux superficielles sur tout le territoire des 28 pays de l'Union Européenne. Pour assurer la protection de la santé humaine et de l'environnement, la réglementation REACh (Enregistrement, Evaluation et l'autorisation des produits chimiques) est également entrée en vigueur le 1er juin 2007 (REACH 2006) et impose aux industries la responsabilité d'évaluer les dangers et les risques liés à l'utilisation des produits chimiques. Comme toute société industrielle, l'entreprise Energie De France (EDF) est tenue au respect de la réglementation environnementale et se doit de conforter l'acceptabilité environnementale de ses installations de production en surveillant l'absence d'impact de ses activités vis-à-vis du milieu aquatique.

Actuellement, l'évaluation du risque environnemental liée à l'utilisation des substances chimiques se résume le plus souvent à la réalisation de tests standards monospécifiques de toxicité aigüe sur des organismes représentatifs de différents taxons et de différents niveaux trophiques (e.g., algues, daphnies, poissons). En raison de sa simplicité, sa rapidité et de son faible coût, le test de toxicité aiguë d'immobilisation sur *Daphnia magna* (ISO 6341, 2012, OCDE 202, 2004) est l'un des tests les plus fréquemment utilisés parmi ces outils standardisés pour évaluer la qualité de l'eau et la toxicité des effluents. L'espèce *Daphnia magna* est sensible à une large gamme de polluants (Martins et al., 2007) et est représentative des organismes d'eau douce (Baird & Van den Brink, 2007, Mark & Solbé, 1998). Cependant, malgré ses avantages, le test d'immobilisation sur *Daphnia magna* présente quelques limites : seul un paramètre d'effet aigu (i.e., immobilisation) est considéré,

et ce à seulement deux temps d'exposition prédéfinis (i.e., 24 heures pour l'évaluation des effluents et 48 heures pour les substances chimiques). Ce test n'apparaît pas suffisamment sensible pour évaluer des effets sublétaux pouvant être induits aux faibles concentrations de contaminants généralement présents dans les eaux de surface. En effet, la contamination des eaux de surface par des concentrations provoquant des effets de mortalité est rare voire accidentelle. Comme l'indique l'étude extensive de 4000 sites de biosurveillance sur le continent européen, seuls 6% des sites étudiés présentent des concentrations maximales dépassant des seuils équivalents à 1/10 de la CE_{50} (48 h) chez la daphnie tandis que 38 % des sites atteignent des seuils équivalents à 1/1000 de la CE_{50} (48 h) (Malaj et al., 2014).

Il apparaît donc nécessaire de développer des indicateurs précoces de perturbations ainsi que des outils permettant de mieux comprendre la contamination chimique de l'eau en termes d'impact pour mieux gérer le risque que représente cette contamination chimique tant pour l'homme que pour l'environnement. Il y a donc une réelle nécessité d'améliorer ou de développer des techniques permettant une détection précoce et une évaluation pertinente des effets écotoxicologiques engendrés par des contaminants à des concentrations environnementales. D'autres paramètres d'effets, autres que l'immobilisation, peuvent fournir des indications sur les effets toxiques des polluants à des concentrations environnementales. Par exemple, les paramètres comportementaux sont de plus en plus considérés comme étant des indicateurs sensibles et précoces de toxicité chez les organismes aquatiques (Coelho et al., 2011).

Les indicateurs comportementaux ont l'avantage de représenter des réponses rapides et sensibles puisque le stress chimique peut induire des réponses rapides au niveau du comportement natatoire chez les organismes exposés à des concentrations inférieures aux seuils de toxicité aiguë (Amiard-Triquet & Amiard, 2013). De ce fait, les indicateurs comportementaux sont bien adaptés pour la détection de concentrations environnementales et pourraient contribuer à améliorer le processus d'évaluation des risques environnementaux (Robinson, 2009). Cependant, l'utilisation du comportement comme critère de toxicité pour l'analyse du risque environnemental ou pour le contrôle de la qualité de l'eau reste, pour l'heure, encore limitée. En effet, il est actuellement difficile de quantifier la sensibilité des paramètres comportementaux et d'établir un lien entre les seuils d'effets comportementaux et les effets aigus et subaigus classiquement mesurés dans les tests en écotoxicologie. De plus, la standardisation des différents profils d'effets comportementaux potentiellement observables et la caractérisation des concentrations des substances chimiques engendrant ces effets n'est pas simple. Pour une évaluation systématique de la sensibilité des paramètres comportementaux chez la daphnie, les substances chimiques doivent être testées à plusieurs

concentrations et réplicats pour permettre une comparaison avec les paramètres standards d'immobilisation. Cependant, la capacité de test des systèmes de mesure du comportement natatoire existants (notamment pour la daphnie) reste limitée et ne permet pas de tester suffisamment de réplicats pour estimer la variabilité biologique du comportement et permettre une comparaison avec le test standard. Actuellement, l'étendue des substances provoquant des effets sur le comportement n'est pas connu, il est donc nécessaire d'explorer les différents profils d'effets comportementaux induits par une large gamme de substances chimiques. En principe, les substances qui possèdent un mode d'action similaire devraient engendrer des effets toxiques similaires et donc des profils d'effets comportementaux similaires (Dom et al., 2012, Zein et al., 2012).

C'est dans ce contexte général que se positionnent ces travaux de thèse avec comme objectifs **(i) le développement d'un nouveau système multi-cuves de mesure du comportement** sur l'espèce modèle ***Daphnia magna*** (ii) de **comparer la sensibilité du test comportemental** par rapport au test standard d'immobilisation et (iii) **d'analyser la pertinence du comportement en tant que bioindicateur de toxicité** pour l'évaluation de la qualité des masses d'eau et des effluents. Dans le but d'améliorer notre compréhension des effets comportementaux des polluants sur *Daphnia magna*, nous avons développé un nouveau système d'analyse du comportement de la daphnie baptisé « Multi-DaphTrack » avec une capacité d'analyses simultanées permettant le suivi de 20 groupes de daphnies exposés à plusieurs concentrations et réplicats simultanément. Ensuite, pour explorer un large spectre d'effets potentiels sur le comportement, douze substances toxiques, couvrant une large gamme de modes d'action différents (i.e., narcotiques, modes d'action spécifiques et multiples), ont été testées dans ce système. Un test standard d'immobilisation ainsi que des analyses de comportement dans le toximètre DaphToxI®, ont également été effectués pour chaque substance afin de comparer les seuils d'effets comportementaux avec les paramètres classiques d'immobilisation.

Après optimisation de conditions expérimentales stables dans le système « Multi-DaphTrack », des analyses en condition témoin ont été réalisées pour mesurer la performance du système. En raison de pertes de reconnaissance des daphnies et d'artéfacts, le taux initial de détection n'atteignait que les 30% lors des premiers essais avec d'importantes variations entre cuves, entre expérimentations et au cours des 48 h d'analyse. L'amélioration du paramétrage de l'installation expérimentale et le réglage des paramètres du logiciel Zebralab ont permis d'améliorer la détection avec un taux de 93%, homogène entre cuves et stable dans le temps. Les résultats des expositions en condition témoin ont permis de sélectionner le

critère d'évaluation le plus pertinent, i.e., la vitesse de nage puisque ce paramètre est plus homogène et stable dans le temps comparé aux paramètres du nombre d'organismes actifs et de la vitesse angulaire.

Les résultats obtenus ont montré que ce nouveau système « Multi-DaphTrack » permet de détecter des effets comportementaux (i.e., augmentation de la vitesse de nage) significatifs et précoces pour l'ensemble des substances testées et ce, à des concentrations proches de la CE_{10} (48 h) du test aigu d'immobilisation dès la première heure d'exposition. Des profils de réponse comportementale différents ont été observés selon les substances testées (i.e., intensité, temps de latence et durée de l'effet). Par exemple, l'exposition aux substances narcotiques (i.e., isopropanol et éthanol) a induit une augmentation intense et rapide de la vitesse de nage, suivie d'un retour progressif au niveau ou en dessous du niveau des contrôles. A l'opposé, les effets sur la vitesse de nage des substances à modes d'action spécifiques ou multiples sont plus hétérogènes et caractérisés par une forte ou légère augmentation de la vitesse de nage suivie d'un retour rapide au niveau ou en dessous des contrôles. De plus, le temps d'apparition des effets sur la vitesse de nage diffère entre les substances à modes d'action spécifiques ou multiples avec une apparition rapide (e.g., fipronil, abamectin, carbofuran, esfenvalerate, cyperméthrine et caféine), intermédiaire (e.g., imidacloprid, sulfate de cuivre et la sertraline) ou retardée (e.g., trichlorfon). Ces profils comportementaux sont contrastés entre les différentes substances testées mais ceux-ci ne sont pas spécifiques d'un mode d'action particulier. Enfin, l'ensemble des résultats obtenus sur le «Multi-DaphTrack » ont permis de montrer que les effets comportementaux débutent généralement au cours des 12 premières heures d'exposition (à l'exception du trichlorfon), et que les effets varient en fonction du temps. Les résultats obtenus avec le toximètre DaphToxI® ont révélé des profils d'effet similaires (i.e., augmentation de la vitesse de nage) bien que ce système soit globalement moins sensible par rapport au système « Multi-DaphTrack ».

Pour conclure, notre nouveau système d'exposition multi-cuves « Multi-DaphTrack » est un outil plus sensible et précoce que le test standard d'immobilisation pour l'évaluation de la toxicité de substances chimiques. L'utilisation du système « Multi-DaphTrack » pourrait donc être envisagée, après quelques améliorations et validations supplémentaires, pour l'évaluation prédictive de la toxicité des substances chimiques et l'évaluation de la qualité des masses d'eau et des effluents.

Mots clés : Comportement natatoire, indicateur de toxicité, *Daphnia magna*, biosurveillance, polluants, modes d'action

REMERCIEMENTS

Ce travail a été réalisé au sein de l'équipe Evaluation des Risques Sanitaires et Environnementaux (ERSE) du Laboratoire National d'Hydraulique & Environnement (LNHE) sur le site EDF-Recherche et Développement de Chatou.

J'exprime ma profonde reconnaissance à Jérôme CACHOT, Professeur à l'Université de Bordeaux, pour avoir dirigé mes travaux de thèse et fait profiter de son expérience. J'ai grandement apprécié son sens de la pédagogie en toute bienveillance.

Je suis très reconnaissante envers Olivier GEFFARD, Docteur à l'IRSTEA de Lyon, Sami SOUISSI, Professeur à l'Université de Lille1 et Joëlle FORGET-LERAY, Professeur à L'Université du Havre, pour avoir accepté de juger ce travail. Je suis également très honorée que Marie-Laure BEGOUT, Docteur à IFREMER de La rochelle, ait accepté d'examiner ce travail.

Je remercie Pascal PANDARD, Docteur Ingénieur à l'INERIS, ainsi que Matthias GROTE, Docteur Ingénieur au département LNHE-EDF R&D, pour leur entière contribution dans l'encadrement de ce travail. Je tiens également à souligner l'étroite collaboration avec Merlin KELLER, Docteur Ingénieur en statistique à EDF-R&D, et je le remercie pour son aide précieuse dans le traitement statistique de nos données écotoxicologiques.

Je remercie la société Viewpoint® Life Technology, tout particulièrement Fréderic Neuzeret, pour sa collaboration dans le développement d'un nouvel outil de mesure selon nos exigences bien spécifiques, un challenge difficile mais que nous avons fini par relever! J'adresse également mes remerciements à Sabine Maestri et aux deux stagiaires Elodie Harcoët et Diane Chadaigne, merci pour votre aide précieuse dans ce travail de thèse et merci pour votre bonne humeur.

Je voudrais remercier d'une manière générale tous les membres de l'équipe du LNHE pour leur soutien et leur bonne humeur : Eric, Matthieu, Aziz, Victor, Said, Rayna, Mohamed, Véronique, Caroline Ferry, Philippe Ciffroy, Pascale, Eloïse, Dominique, les 2 steph', Mélanie, Julie, Hacène, Emilie, Norinda, Samy, Carole, Germain, sans oublier Nico et Sophie de l'IRDEP et Estelle du MRI. Merci à Stéphan pour avoir « méber » la Daphnie. J'ajoute un merci spécial pour Mireille, pour ton soutien et ton sourire à toute épreuve et aussi à « Rachidou » et Aurélie. Je remercie aussi mes amis: Sandra, Sophie, Christophe, Zhanna Tairova du Danemark et ma famille, particulièrement ma sœur Virginie pour m'avoir soutenu dans les derniers moments.

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ABBREVATION

Ach: Acetylcholine

AchE: Acetylcholine Esterase

BEWSs: Biological Early Warning Systems

Cu: Copper

EC_x: Effect Concentration generating an effect for x% of the test population

ERA: Environmental Risk Assessment

GC-MS: Gas Chromatography-Mass Spectrometry

K_{ow}: Octanol-water partition coefficient

LOEC: Lowest Observed Effect Concentration

MEC: Measured Effect Concentration

MoA: Mode of Action of a toxic

NOEC: No observed effect concentration

OECD: Organization for Economic Co-operation and Development

OP: Organophosphorus Pesticide

PEC: Predicted Environmental Concentration

PNEC: Predicted No Effect Concentration

QSAR: Quantitative Structural-Activity Relationship.

REACh: Registration, Evaluation and Authorization of Chemicals

TGD: Technical Guidance Document

USEPA: United States Environmental Protection Agency

WFD: Water Framework Directive

GENERAL INTRODUCTION

Since the beginning of the industrial revolution, the environment (atmospheric, terrestrial and aquatic) has suffered of an increasing anthropogenic pressure. The exponential development of industrial, urban and agricultural activities has generated the release of numerous substances into the environment. These compounds often end up in the aquatic environment and consequently threat aquatic ecosystems. The aquatic environment is mostly contaminated with pesticides used in agriculture, chemicals such as polycyclic aromatic hydrocarbons or heavy metals from industrial and urban discharges, or emerging substances such as pharmaceuticals released by both hospital and pharmaceuticals industries. For instance, a recent large-scale study encompassing 4000 European monitoring sites, revealed that nearly half of European water bodies are subjected to organic chemical pollution (Malaj et al. 2014). This diffuse chemical pollution of aquatic compartment may lead to human health problems but also may impact the aquatic ecosystem, threatening biodiversity and hence the balance of aquatic ecosystem.

Facing the growing degradation of the quality of European waters, the European Union (EU) has responded by adopting, on October 23, 2000, a directive establishing a framework for Common action in the field of water, called **Water Framework Directive** (WFD 2000). The aim of this directive is to achieve good qualitative and quantitative status of all water bodies (ground and surface water) by 2015. More recently, the regulation **REACh** (Registration, Evaluation and Authorization of Chemicals), has entered into force on 1st June 2007 (REACH 2006) to ensure the protection of the human health and the environment and to make industry responsible for assessing and managing the risks that can be posed by chemicals. As all industrial facilities, the French company "**Electricité de France**" (EDF) must meet the requirements set by these environmental legislations in order to verify the absence of impact of its activities on the environment.

When assessing the hazard and the risk of chemicals released into the aquatic environment, potential toxicity is usually evaluated using **standardized toxicity tests** on representative organisms from different trophic levels (fish, daphnia and algae). The **immobilization test on *Daphnia magna*** (ISO 6341 2012, OECD 2004) is one of the most frequently used standardized tests for assessing the hazardousness of chemicals and monitoring water quality. However, the acute standard test does not consider other endpoint than immobilization at two pre-defined exposure time (i.e., 24 and 48 h) while for protecting the aquatic ecosystem, a rapid assessment of the toxic potency of environmental pollutants is needed. Furthermore, in some effluent toxicity and in many surface water assessments, *Daphnia magna* immobilization is not a suitable tool to measure water quality since sub-lethal effects can occur at much lower concentrations. Indeed, concentrations inducing mortality are fortunately rarely reached in the aquatic environment and a recent large-scale study encompassing 4000 European monitoring sites revealed that for only 6 % of measured sites, maximum concentrations of organic

chemicals exceed 1/10 the EC_{50} (48 h) level for *Daphnia magna* while for 38% of sites, maximum concentrations exceed 1/1000 of the EC_{50} (48 h) (Malaj et al. 2014).

The consideration of other endpoints, such as **swimming behavior,** may provide earlier responses compared to both acute/chronic standard tests since toxic stress can induce rapid behavioral changes in exposed organisms at concentrations well below the acutely toxic levels (Amiard-Triquet and Amiard 2013). To protect the aquatic ecosystems, behavioral endpoints represent good candidates as "early warning" signal of toxicity (Hellou 2011). Furthermore, behavior is a sensitive indicator of acute/sub-lethal toxicity (Coelho et al. 2011) and is suited to detect stress induced by realistic environmental concentrations of pollutants. The use of data on behavioral toxicity could consequently improve the evaluation process of environmental risk assessments (Robinson 2009). Gradually, systems measuring organism's behavior have been developed and used to complement conventional water quality monitoring approach (e.g., chemical analysis and toxicity assays), as this latter is less adapted to *in situ* or continuous detection of pollutants in the environment (Butterworth et al. 2007, Osbild et al. 1995). Indeed, current methods measuring levels of contamination in water face the limits of analytical method sensitivity, but also the difficulty of integrating the impact of many environmental biotic and abiotic factors. Furthermore, facing the huge amount of chemicals potentially present in the environment, the screening of all aquatic pollutants is not realistic. Besides, in the environment, toxicants commonly occur in mixtures rather than as single compounds.

O**n-line biomonitors**, so-called biological early warning systems, measure in **real time continuous** information on behavioral stress responses of test organisms exposed to ***in situ*** medium (Gerhardt et al. 2006). These biomonitors typically use representative test organisms from different trophic levels: bacteria, algae, water flea, mussel and fish (Osbild et al. 1995) and can be directly used on the field providing a more representative vision of realistic environmental situations, in contrast to conventional standard tests that involve an artificially controlled environment. The technological advances have allowed developing sophisticate systems, which require low maintenance and provide continuous information automatically. After the well-known incident of Sandoz agrochemical storehouse's fire in Basel in 1986, the runoff has contaminated Rhine River waters with tons of pesticides and has impacted much of the surrounding living organisms including massive fish kills. Following this highly mediatized environmental disaster, the "Rhine Action Program" has been created by several international cooperation, and innovative early warning biomonitoring systems like algae, daphnia, mussels and fish toximeters have been installed in the Rhine and the Meuse rivers located at the German and Belgian border (Hendriks and Stouten 1993). To date, on-line biomonitors are daily used as alternative or complement to conventional water quality monitoring approaches (e.g. chemical analysis and toxicity assays) and in some case it has even been able to detect pollution which was not detected by traditional chemical analysis systems (De Hoogh et al. 2006). For instance, the daphnia toximeter DaphTox® (Bbe® Moldaenke, Kiel, Germany) is successfully used for 15 years as

an early warning system for detecting chemical pollution in surface water or intakes for drinking water production in northern Europe (Werth 2006) or in rivers affected by industrial activities in China and North Korea (Van Den Broeke 2013).

This study takes place in the works conducted by the **National Laboratory of Hydraulics and Environment (LNHE, EDF R&D)**, aiming to study the influence of chemicals on the environment. **EDF** has partnered with **INERIS** (National institute of industrial environment and risks, French public agency) in order to develop a new system for continuous water quality measurement by using behavior of living organisms as toxicity bioindicators. This new system is a **mobile station** that can be placed *in situ* and can operate continuously and autonomously for evaluating in **real-time** the quality of the natural aquatic environment. This mobile station is constituted of three biological early warning systems BEWS (so-called "toximeters", Bbe® Moldaenke, Kiel, Germany), which continuously measure the behavior of organisms from different trophic levels (i.e., micro-algae, daphnia and fish) exposed to the medium of interest. The water is pumped and flows into a settling tank including a sensor measuring pH, temperature, and conductivity and dissolved oxygen from the incoming water, which is then distributed to the 3 toximeters. When a change in the behavior is observed, an alarm is triggered and water sampling is started to allow further chemical analysis. In this way, toxic information is provided in real time at the moment of actual pollution occurrence and water sampling allow further chemical analysis to determine the toxic that cause behavioral changes.

To date, the **mobile station** can be considered as functional and operational but scientific validation is still needed before its use in environmental assessment. First of all, the relevance of the use of on-line biomonitors in water survey context is directly linked to the studied species: the algae toximeter is more specific for the determination of herbicides, whereas daphnia and fish toximeter are sensitive to an extended type of contaminants. Besides, daphnia behavior tends to be more sensitive for short term monitoring compared to fish (Ren and Wang 2010). For the reasons mentioned above, we have decided to focus on the daphnia toximeter in this study. Alarm threshold concentrations inducing detectable changes in daphnia behavior have been established for numerous compounds and are used as reference by Daphnia toximeter operators (Lechelt 2006). However, no systematic analysis has yet been performed, and the results are highly dependent on the test conditions. With their limited test capacity these systems do not provide enough information to improve our understanding of the toxic effects of pollutants. Consequently, the link between the information related to the behavior of organisms and water quality is yet not clearly defined. Before considering the possible use of the daphnia toximeter in environmental assessment, a thorough understanding of behavioral effects under single chemical exposure in controlled conditions (i.e., laboratory) is first needed for a reliable utilization of behavior as an ecotoxicological endpoint and for the good behavioral data interpretation.

So far, systematic understanding of behavioral effects and comparability between effect profiles is hampered, as available studies are limited to few chemicals, typically selected for a specific assessment purpose which differs in exposure conditions and effect parameters. Furthermore, standardization and field validation of these behavioral responses are still lacking and the characterization of the chemical concentrations that can induce behavioral effects is not straightforward. For a systematic assessment of the sensitivity of behavioral endpoints in daphnia, chemical substances have to be tested with different concentrations and sufficient replicates, allowing a comparison with standard endpoints. However, among all existing behavioral analysis systems, their limited test capacity does not allow to perform enough replicate numbers to understand biological behavior variability and compare this to a standard test. Moreover, it is still unclear what extent of chemicals lead to behavioral effects, thus an investigation of the different behavioral effects profiles induced by a large range of chemicals must be conducted. The selection of testing chemicals by their mode of action may potentially facilitate extrapolation of observed behavioral effects profiles and time of effect onset induced by toxic chemical exposure, as we can postulate that compounds with a similar mode of action should behave, in a similar manner, showing similar toxic effects and thus similar behavioral effects (Dom et al. 2012, Zein et al. 2013). The **aim of this thesis work** was (i) **to develop a laboratory behavioral analysis system with high capacity** allowing behavior tracking of daphnia groups exposed to several concentrations and replicates simultaneously (ii) **to determine the sensitivity of the behavioral parameter** compared to the standard immobilization endpoint and (iii) to **analyze the relevance of behavior** as a toxicity bioindicator in the context of water quality monitoring and effluent toxicity assessment.

The manuscript is structured around four chapters. The **chapter I** provide a literature review presenting biological information of the species *Daphnia magna* and its use in risk assessment. A second part broaches the measurement of behavior and gathers the variety of the existing behavioral analysis systems and their applications in water quality assessment. To support the experiences, an overview of the selected chemical modes of action is given. The development of our new multi-cell exposure system "named Multi-DaphTrack" is described in the **chapter II** and an example of application is broached by testing the insecticide esfenvalerate in this new developed system and comparing results with the acute standard test and the commercialized single-cell biomonitor DaphTox®. **In the chapter III,** different daphnia behavioral effects profiles have been explored by testing a broad range of toxicants with different modes of action. The understanding of these effects is discussed according to the mode of action of the compound and the sensitivity has been evaluated for all chemicals tested in regard to acute standard test and the DaphTox®. Finally, a synthesis of all the results of this study is presented in **the chapter IV** and a possible application of the new multi-cell exposure system in risk assessment is also discussed and put in perspectives.

CHAPTER I. BIBLIOGRAPHIC CONTEXT

1. INTRODUCTION TO ENVIRONMENTAL RISK ASSESSMENT

According to the technical guidance document on risk assessment of the European commission (2003), environmental risk assessment evaluate the likelihood that the environment may be impacted by the exposure of one or more environmental stressors such as chemicals. This approach involves four step processes:

- Hazard identification;
- Dose-response (effect) assessment;
- Exposure assessment;
- Risk characterization.

The hazard identification consists in examining toxicity of a chemical on model organisms. To assess the dose-response relationship of a chemical, toxicity measurements are usually performed using **standardized toxicity tests** on representative organisms from different taxa and trophic levels. Toxicity data are then extrapolated to predict the likely effect of low doses of the chemical. The exposure assessment is performed by measuring or predicting magnitude, frequency and duration of exposure to a chemical. The potential for risk to an exposed individual or population is then characterized. Predicted No effect Concentration PNEC can be calculated using values from standardized toxicity tests by dividing the toxicity threshold obtained with the most sensitive assay with an assessment factor. This assessment factor depends on the number (trophic levels assessed) and type (acute or chronic data) of data available. For instance, when only short-term toxicity data are available, the assessment factor used is high (1000). Then, the more data are available, the lower is this factor. The calculated PNEC is then compared to the PEC (Predicted Environmental Concentration) or the MEC (Measured Environmental Concentration) to characterize the risk of the chemical for the environment.

1.1 *Daphnia magna*, a model organism for risk assessment

Along with Fish and Algae, Daphnia is the backbone test species of acute and chronic standards tests in environmental risk assessment of chemicals. The freshwater crustacean *Daphnia magna* has been central to many researches in diverse areas of biology (Peters and De Bernardi 1987) and has become a model organism for ecotoxicological bioassays. The selection of a species as bio-indicator is often done in function of their **ease to culture**, their **sensitivity** to stresses and their predictable way of **responses** to stresses (Goodsell et al. 2009). Daphnia exhibits a number of natural responses to environmental stress (physiological, biochemical or behavioral responses). Raising daphnia can be achieved in a small space and its short life cycle and its high fecundity make it easy and economical to culture in the laboratory. Daphnia's carapace is transparent, which facilitates observation of internal organs and hence measurements. With parthenogenetic reproduction, brood from any female is genetically identical to their mother and this ensures less variability in results, especially variability in behavior, which can be high. *Daphnia magna* is representative of freshwater

organisms (Mark and Solbé 1998) since this crustacean cladoceran occupies a central position in aquatic food web and is often one of the dominant zooplankton species present in freshwater lakes or ponds (Duquesne and Küster 2010). Daphnia plays an important role in maintaining the ecological balance of aquatic ecosystems: a variation in its population may cascade to phytoplankton communities and consequently can worsen the impact of eutrophication or algal blooms or it can also lead to implications within the aquatic food webs (Riddell et al. 2005).

An important number of data from standard acute tests with *Daphnia* spp., *Pimephales promelas* (fathead minnow), and *Mysidopsis bahia* (Mysid) and chronic tests with *Pimephales promelas*, *Ceriodaphnia dubia* (water flea) and *Cyprinodon variegatus* (Sheepshead minnow) is available. Nevertheless, **the most extensive data** found in laboratory on single chemical toxicity and in whole effluent toxicity are from the **acute test with *Daphnia* spp.** (Malaj et al. 2014, Parkhurst et al. 1992). Thus, the selection of this species for behavioral tests may allow comparison with risk assessment database. *Daphnia magna* is highly sensitive to a wide range of chemicals (Mark and Solbé 1998) but there is no general rules that daphnia is more sensitive than other species (such fish or other invertebrates). Indeed, the species sensitivity is strongly dependent of the type of chemical tested. For instance, by comparing acute toxicity data, daphnia was found to be more sensitive to organophosphates, but less sensitive to neonicotinoids compared to another invertebrate *Gammarus pulex* (Ashauer et al. 2011). The evaluation of acute toxicity databases showed that *Daphnia magna* respond to a larger variety of chemicals with a higher sensitivity compared to the fish *Danio rerio* (Martins et al. 2007). We may also expect higher sensitivity of *Daphnia magna* compared to fish in behavioral assays. This was already the case where in a previous study, the sensitivity of *Daphnia magna* was about two orders of magnitudes higher than that of Japanese Medaka in different types of toxic chemicals (dichlorovos, deltamethrin and cadmium chloride) (Ren and Wang 2010). It is noteworthy that fish behavior is highly influenced by external factors (available food, temperature and light) which make their behavior much more variable and complicated to control. For all these reasons, daphnia appears as a reliable model species for ecotoxicological and behavioral studies.

1.2 Standard tests on *Daphnia magna*

Two types of toxicity tests on *Daphnia magna* can be distinguished (see Table 1).

The **acute toxicity test** on *Daphnia magna* is realized on a short period compared to the lifespan of the organism and consists in observing the immobilization at 24 and 48 H of exposure. The immobilization test on *Daphnia magna* (ISO 6341 2012, OECD 2004) is one of the most used standardized tests for assessing hazard of chemicals and monitoring water quality because this test is simple, rapid, and cost-effective. The concentration inducing 50% of immobilization (EC_{50}) is calculated from the dose-response curve. However, the concentrations producing acute effects are generally high and exceed the environmental concentrations.

The **chronic toxicity test** on *Daphnia magna* (OECD 2012) consists in assessing the effects of chemicals on the reproductive output of *Daphnia magna*. This test is realized on a relatively long period (21 days) compared to the life span of the organism, effects are observed on a more sensitive endpoint (i.e., reproduction) compared to the immobilization endpoint of the acute test and provide long-term results. Female Daphnia neonates, aged less than 24 hours at the start of the test, are exposed to the test substances added to water at a range of concentrations. After 21 days of exposure, the total number of living offspring produced is assessed. The toxic effect of the test substance on reproductive output is expressed as Effect Concentration EC_x by fitting the data to an appropriate model by non-linear regression to estimate the concentration that would cause x % reduction in reproductive output, respectively, or alternatively as the NOEC (No Observed Effect Concentration)/LOEC (Lowest Observed Effect Concentration) values.

Table 1: Condition of the two different standard toxicity tests with *Daphnia magna*:

Standard test	Duration	Experimental condition	Feeding	Parameter observed	Endpoint	Norm
Acute	24 h 48 h	No light Static	No	Immobilization	EC_{50}	(ISO 6341 2012, OECD 2004)
Chronic	21 days	No light Semi-static Flow-through conditions	Yes	Reproduction	LOEC NOEC	(OECD 2012)

The life stage plays an important role in toxicity determination: the early life stage is more sensitive than the adult life stage to chemical toxicants, partially because of their higher surface-area-to-volume ratio and hence more rapid rates of toxicants uptake. For instance, neonates show higher sensitivity than 7 days old organisms (Muyssen and Janssen 2007). That is why; both **acute and chronic** are performed on daphnia neonates (less than 24 hours). The chronic test is much more sensitive than the acute test. However, the chronic test is more time consuming and expensive compared to the acute test.

2. *DAPHNIA MAGNA* BIOLOGY

Daphnia magna is a micro crustacean that belongs to the Branchiopoda, usually known to produce water current from its filtering apparatus (see Figure 1).

Phylum: Arthropoda
Subphylum: Crustacea
Class: Branchiopoda
Suborder: Cladocera
Family: Daphniidae
Genus: *Daphnia*
Species: *Daphnia magna Straus, 1820*

Figure 1: Taxonomy of *Daphnia magna* and picture of an adult female from our laboratory:

This planktonic cladoceran is widely spread in standing freshwater from temperate climate areas: it can be found in ponds and lakes, rivers and streams, temporary pools or in brackish water in Western Europe or along the North-eastern United States with optimum temperatures ranging from 18 to 22°C although they can tolerate a much broader temperature range (Dieter 2005, Vanoverbeke et al. 2007, WoRMS 2012).

2.1 Physiology and metabolism

The body length of *Daphnia magna* ranges from 3 to 5 mm at the adult life stage*;* its anatomy is illustrated in the Figure 2 and Figure 3. The body is divided into two parts: an anterior **head** and the **thorax**.

- The **head** smoothly rounded and bent on the ventrally consists of the oscellus, i.e., a compound pair of 2 lateral fused eyes, the mouth and 2 antennas arisen on each side of the head, which serve for the locomotion. Each antenna consists of a single basal so-called peduncle, controlled by muscles and two rami arising from the distal end of the peduncle. The two rami bear large plumose swim setae.
- The **thorax**, which makes up most of the remainder of the body, contains six thoracic appendages, which draw water through a setae filter to carry food and oxygen to their mouths and gills. Food particles are stopped by this setae filter and retained in the ventral food groove to be then directed to the mouth. The heart is situated in the dorsal region of the anterior thorax. The gonads are located on either side of the intestine track; this latter crosses the whole body from top to bottom. In females the ovary opens dorsally, via an oviduct, into the brood chamber. Female have a large brood pouch located under the dorsal carapace in which eggs are brooded until they complete embryonic development and become juvenile cladocerans. The abdomen is composed of the anus at the posterior tip and of a pair of abdominal claws which aiming to clean the thoracic appendages (Dieter 2005).

The whole body is protected by a transparent carapace, mostly made by chitin and renewed at each molting. At the adult life stage daphnia molt every 2 or 3 days. At the juvenile life stage, the

growth is very important and molting is much more frequent since the growth is occurring after each molt when the carapace is still elastic. The inferior part of the carapace ends by an apical spine whose function may be to interfere with predation (Hazanato 1999). The daphnia nervous system consists of the brain and ventral nerves, which connect the brain to the appendices and the body of the daphnia. The brain is composed of three different parts which are each dedicated to a particular part of the body: the "protocerebrum" (for eyes, optical neuropils), the "deutocerebrum" (sensorial antenna) and the "tritocerebrum" connected to antenna, nabral nerves and intestines (Weiss et al. 2012). Daphnia have an open blood circulation and extracellular respiratory proteins hemoglobin (Hb) in order to ensure oxygen transport. They are able to absorb ions via chloride-absorbing glands and the excretion and osmoregulation is controlled by shell gland.

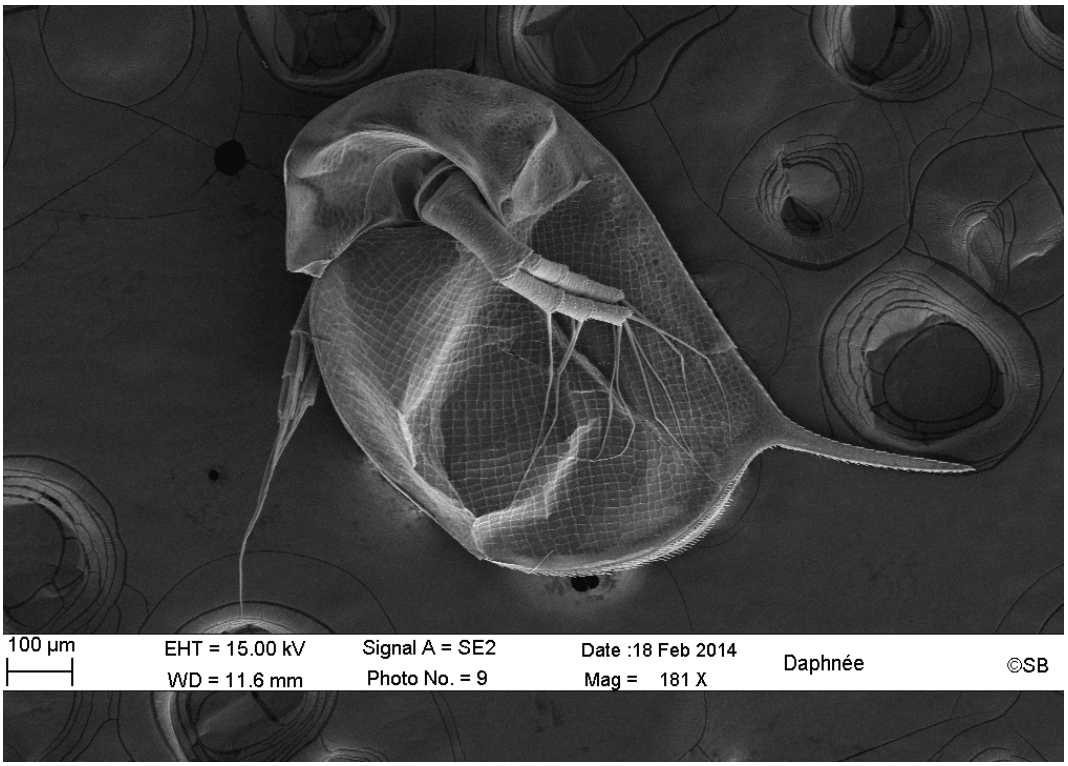

Figure 2: Scanning electron microscopic of an adult female *Daphnia magna* (picture of Stephan Borensztajn, EDF R&D-IRDEP department).

2.2 Nutrition

This small planktonic cladoceran is a filter feeder of small-suspended particles in the water (<50 µm in diameter), mostly composed of primary producers (i.e. algae, yeast, and bacteria). Daphnia is consumed by zooplanktivorous fish and by other invertebrates, especially the larvae of the phantom midge *Chaoborus*.

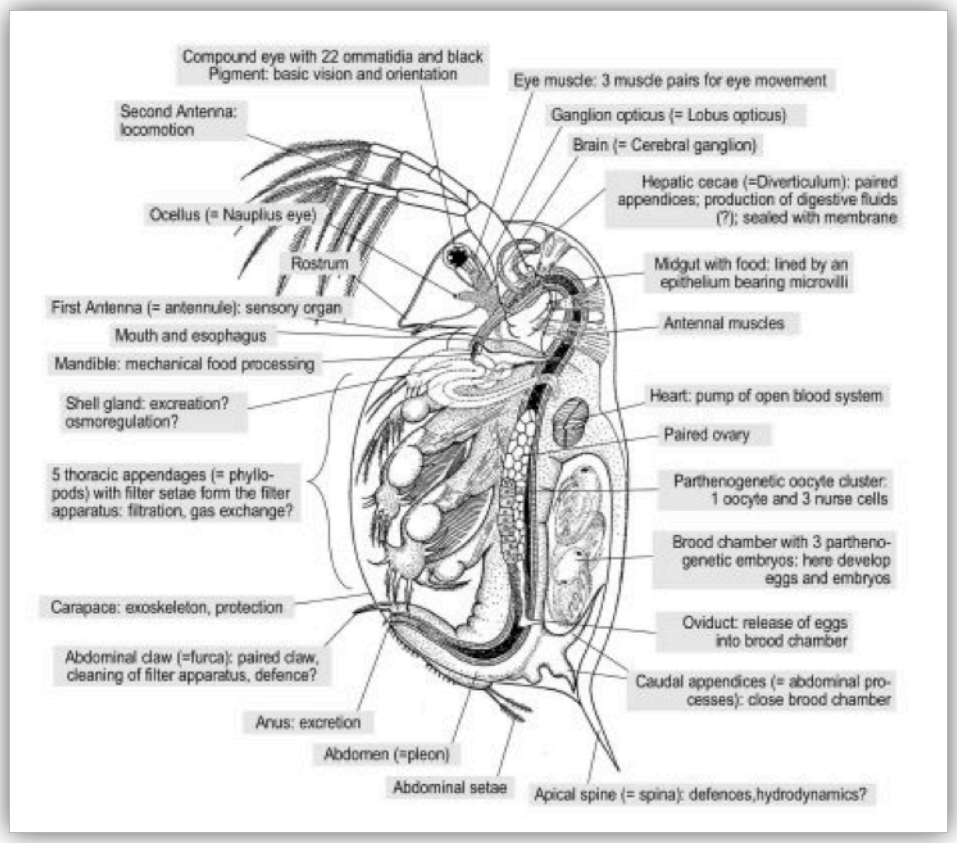

Figure 3: Physical description of *Daphnia magna* at adult life stage (Dieter 2005).

2.3 Development & reproduction

Four distinct periods may be recognized in the history of the life of *Daphnia magna*: (1) embryo, (2) juvenile, (3) youth and (4) adult. The development of an embryo from parthenogenetic reproduction follows 6 distinct developmental stages, which takes place in the mother brood chamber (see Figure 4):

- **Stage 1:** 6 hours post fecundation (pf), the embryo is perfectly spherical, when embryonic differentiation has not occurred.
- **Stage 2:** 12 h pf, early cell differentiation occurs and the embryo becomes asymmetric.
- **Stage 3:** 24 h pf, the body surface and oscellus (i.e., compound pair of 2 lateral fused eyes) have formed gradually.
- **Stage 4:** 36 h pf, early embryonic maturation, the head and the secondary antenna are differentiated and the tail spine has formed without breaking away from the tail end.
- **Stage 5:** 48 h pf, the Malpighian tube has matured with straight tail spine and the second embryonic membrane is broken.
- **Stage 6:** growth at 60 h, the first and second newborn antennas have developed completely, embryos develop at 72h into mature zooids and the body begins to swim.

The mother releases the neonates through ventral flexion of the post-abdomen. An adult female can produce eggs every 2 days until death when environmental conditions are favorable. Generally, a range of 6 to 12 eggs is deposited into the pocket brood, but the number can reach up to 60 eggs. The rate of growth of organisms is important during the juvenile stage and size body can double after each phase characterized by the end of molting given the elasticity of the new shell. Then, the young stage is very short since Daphnia becomes sexually mature after 5 to 10 days (first reproduction cycle). In laboratory, daphnia may live for 6 to 8 weeks (Grzesiuk et al. 2010). However, the lifetime increases with decrease in temperature due to the decrease of metabolic activity: 40 days at 25 ° C and 56 days at 20 ° C. *Daphnia magna* usually has between 6 to 22 spawning cycles in adulthood but the number of offspring per spawning is highly variable depending on the food availability and environmental conditions.

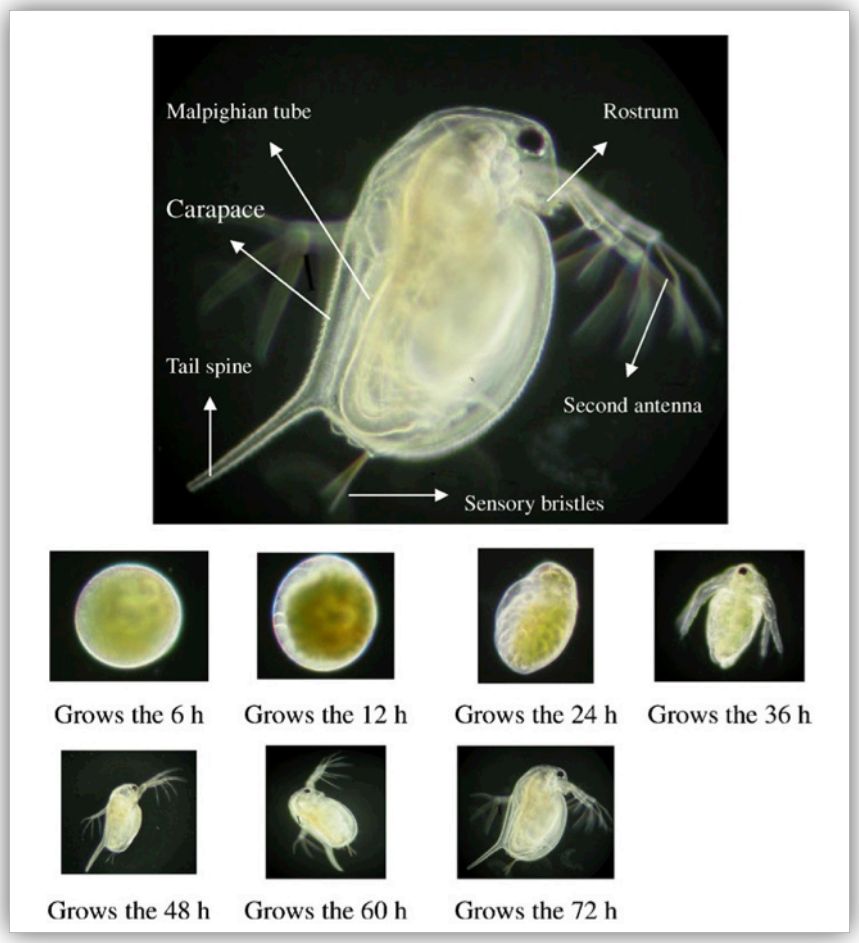

Figure 4: Picture of *Daphnia magna* neonate and the different early development stages (Wang et al. 2011).

Males and females can be distinguished mainly by their size, color and moving speed. Indeed, males are smaller than female, have a bigger first antenna pair, are orange and swim much faster than females. The reproduction of Daphnia species follows a cyclic parthenogenesis, which involves the alternation of two different phases depending on the environmental conditions, i.e. asexual and sexual

phases (see Figure 5) (Dieter 2005, Kleiven et al. 1992). Under favorable conditions, females reproduce following an asexual mode (by parthenogenesis) by producing exact clones of females (diploid eggs which are directly developed in the mother's brood chamber). In laboratory, females reproduce by parthenogenesis in favorable conditions. On the contrary, sexual reproduction occurs during adverse conditions (low food availability, extreme temperatures or high population density). Sexual phase reproduction is initiated by male offspring production and the production of resting eggs by females (haploid eggs), which, after being fertilized by males, enter in embryonic diapause encased in ephippia (very resistant eggs) until conditions become favorable (Kleiven et al. 1992). In laboratory, females are generally cultivated in conditions favoring reproduction by parthenogenesis and may live for 6 to 8 weeks.

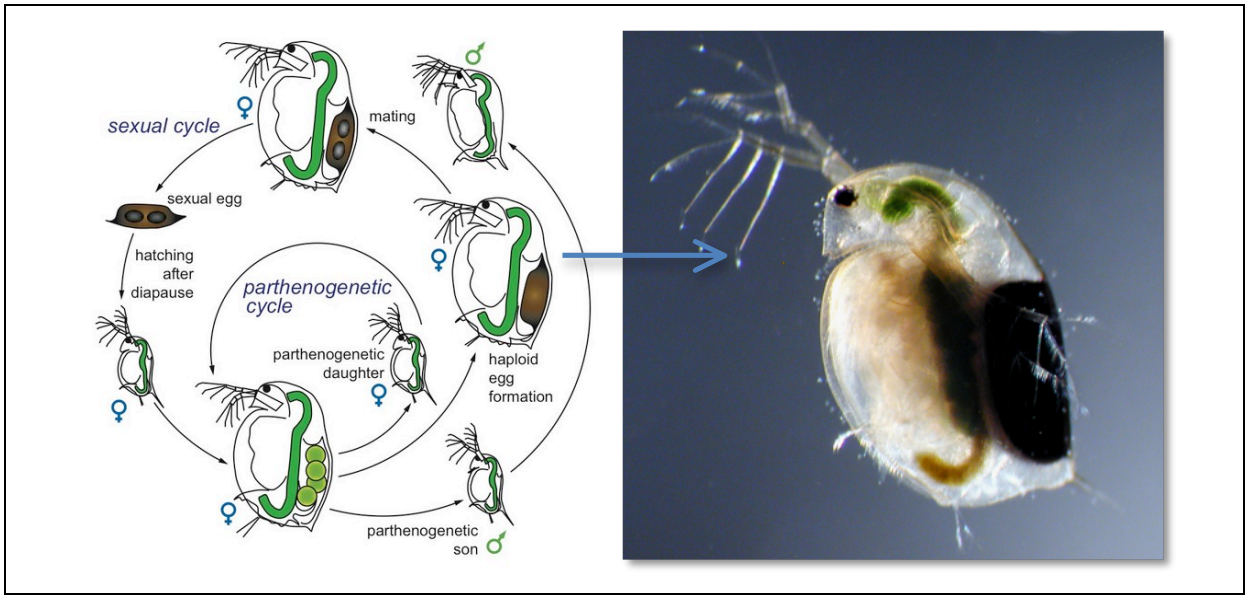

Figure 5: Life cycle of a cyclic parthenogenetic of Daphnids, drawing by Dita B. Vizoso, Fribourg University (Dieter 2005) and a picture of an adult female with ephippia, which can be easily recognized by its dark color.

2.4 Behavioral ecology

Daphnia are commonly called water fleas because of their jumping-like behavior inducing a jerky swimming. Their locomotion is characterized by small antennae downbeats which allow a quick upward movement but when Daphnia stop to swim, they sink rapidly to the ground (Dieter 2005). This organism is mostly pelagic (i.e. found in the water column). However, Daphnia behavior is governed by diel vertical migration: a migration toward upper levels of the water column during night time and then back downward during the early morning and day time. Diel vertical migration is induced by phototaxis (Ringelberg 1964) but also reflect both combined effects of predator and starvation avoidance (Dawidowicz and Loose 1992, Stirling and Roff 2000). Indeed, during daylight, daphnids hide from fish in down water levels since they are less visually detectable by predators due to low light intensity. However, food availability is low in down water levels, hence, during the night, daphnids

take advantage of the richer food (planktonic algae) in the upper water levels. Another typical behavior may be observed when food is limited: daphnids may cling to substrates or plants over the surface or the bottom for searching food. Daphnids can adjust their swimming speed in function of algae concentration present in the medium (decrease of the swimming speed at high algae concentrations). Daphnia seems to possess olfactory senses allowing predators avoidance (Roozen and Lürling 2001). Chemically induced anti-predator defenses is well known in planktonic organisms, diverse morphological, behavioral and life-history responses have been observed and induced by the chemical presence of potential predators (Lass and Spaak 2003). Daphnids have been reported to be able to detect the presence of potential predators through kairomone, an infochemical inducing generally a behavioral or physiological reaction (De Meester 1993). Escape behavior, i.e., increase of the average speed, has been observed for *Daphnia pulicaria* in the presence of fish (Brewer et al. 1999). When comparing the predator-induced life history responses and morphological transformations to behavioral responses on the time scale, the latter obviously allow the most rapid (almost immediate) predation defense. For instance, rapid phototactic behavioral changes were observed in *Daphnia magna* exposed to fish kairomones (Boersma and Spaak 1998, De Meester and Cousyn 1997). Changes in phototactic behavior, aggregation behavior and escape abilities can also be induced by damaged conspecifics (Pijanowska 1997, Pijanowska and Kowalczewski 1997). These responses are assumed to be an adaptive reaction to reduce predation risk. According to (Szulkin et al. 2006), uniformity in swimming behavior may be an important defense mechanism in zooplankton, aiming to not draw attention among group members and hence decrease predation risk. Another study shows that the patchiness of *Daphnia pulex* increases in the presence of fish or fish kairomones (Jensen et al. 1998). Such aggregation and swarming are done to confused predators with the number of preys.

2.5 Population dynamic

Except competition for food between individuals, there is no social hierarchy among the species *Daphnia magna*. Daphnia used to stay in groups of general high density although the population may strongly vary in density through seasons (Dieter 2005).

3. THE USE OF BEHAVIORAL ENDPOINTS FOR RISK ASSESSMENT

3.1 Why study behavior?

The efficient monitoring of water resources is fundamental for effective management of water quality and aquatic ecosystems. To meet the objectives set by the Water Framework Directive (WFD 2000), efficient water quality biomonitoring is required to control water quality in aquatic ecosystems (i.e. surface water, ground water, effluent and drinking water). In the past, water quality biomonitoring

was usually performed with spot samplings for chemical analysis and several physicochemical factors: pH, dissolved oxygen and biochemical oxygen demand. However, a priori knowledge about the type of substances to be monitored is required before assessment. Beside, physicochemical biomonitoring systems cannot detect all concentrations of all chemicals present in the environment and do not provide information on toxic effects induced on aquatic ecosystems. Furthermore, mixtures of pollutants currently occurred in the environment, whose effects may not be predictable only on the basis of chemical analyses. Current chemical analysis systems (e.g. GC-MS, HPLC) can detect accurate concentrations of a selected number of chemicals (up to traces) but measurements are limited because of technical and high cost reasons. Furthermore, chemical concentrations in the environment may highly vary over time and spot samplings reflect only chemical pollution at a given time. Hence, one can miss out the real pollution peak. Even in prospective assessment of water quality, it is important to discover effects related to chemical substances before that significant effects occur on population level (Figure 6). The consideration of other endpoints, such as **behavior,** may provide earlier responses compared to both acute/chronic standard tests since toxic stress can induce rapid behavioral changes in exposed organisms at concentrations well below the acutely toxic levels (Amiard-Triquet and Amiard 2013).

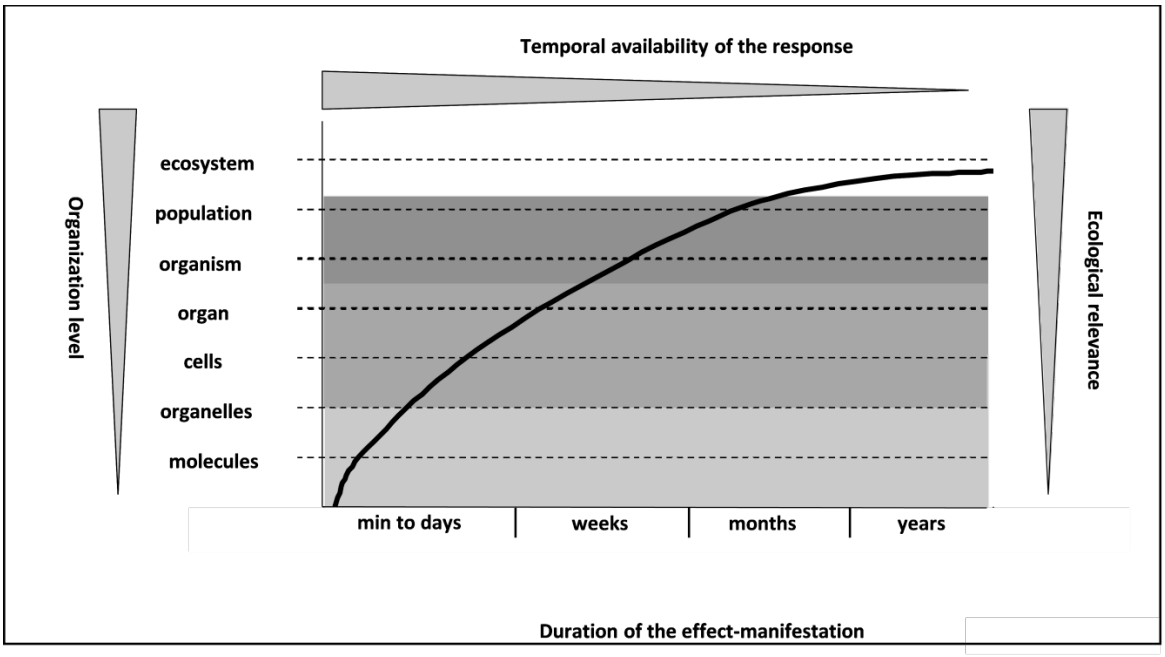

Figure 6: Response time and ecological relevance of different levels of biological organization (Bae and Park 2014)

3.2 Ecological relevance of behavioral tests

Instead of immobilization, the reduction of the overall organism's health can provide useful information on induced adverse effects and allow a toxicity threshold to be determined. Significant impacts (e.g., altered behavior, growth and reproduction, etc.) can occur below the median effective concentration (EC_{50}) level (Dodson and Hanazato 1995). To protect aquatic ecosystem, a rapid assessment of the toxic potency of environmental pollutants is needed. Behavior changes may be a sensitive indicator of acute/sub-lethal toxicity (Coelho et al. 2011) and is especially suited to detect

stress induced by realistic environmental contaminant concentrations. According to Amiard-Triquet and Amiard (2013), behavior can be described as a key link among cascading effects from infra to supra-organismal responses. Indeed, various biochemical and physiological impairments (e.g. on neurotransmitters, enzymes, hormones and energy metabolism) may lead as a consequence to behavioral effects. Behavior change is an endpoint of a sequence of different neurophysiological processes (Lagadic et al. 1994) and can be considered as an integration of physiological, sensorial, nervous and muscular systems (Charoy et al. 1995). Behavioral tests provide early and intermediary responses prior to the death of organisms since toxic stress can induce rapid behavioral changes in exposed organisms (Amiard-Triquet and Amiard 2013). Furthermore, behavior analysis is not expensive (low cost), practical (low maintenance) and non-destructive which allows to subsequently carry out other biological or chemical analyses. It also represents a good alternative to decrease the number of sacrificed animals as it is recommended in REACH regulation (REACH 2006). From another perspective, significant alterations in behavior can also impact the overall state of health and survival of organisms, which may lead to long-term changes at population and community levels (Duquesne and Küster 2010). Indeed, in case of accelerated swimming, energy for normal metabolic functions (e.g., growth, reproduction and locomotion) is reallocated for locomotion and thus may impact long-term survival (Knops et al. 2001). Moreover, adaptive behavior such as avoidance or alteration of motility under toxic stress can impact decision-making (e.g. location), making organisms more noticeable to predators and thus potentially increasing their predation.

3.3 Behavioral studies

Since the 80s, fish swimming behavior has been more and more studied in order to evaluate environmental stress and behavioral signs of stress, such as average speed increase (i.e., escape of the contaminated area, so-called “chemical avoidance”) in case of metals (Scherer and McNicol 1998), pesticides (Floyd et al. 2008) or pharmaceuticals exposure (Painter et al. 2009). Gradually, swimming behavioral studies extend to other aquatic species such invertebrates. For instance, significant alterations of daphnia swimming parameters have been reported after exposure to xenobiotics such as metals (Jeon et al. 2008, McWilliam and Baird 2002, Untersteiner et al. 2003), pesticides (McWilliam and Baird 2002, Zein et al. 2013), nanoparticles (Artells et al. 2013)), water field samples (Barata et al. 2007) and cyanobacteria (Ferrao-Filho et al. 2014). Under stress conditions, behavioral changes such as escape (increase of the swimming activity), adaptation (use of energy for adaptive mechanisms) or protection reaction (decreased swimming activity) can be observed (Wolf et al. 1998). Those significant alterations of the mobility can not only impact the fitness and survival of organisms but also lead to long-term changes at the population and community levels (Duquesne and Küster 2010). Sub-lethal effects occurred at concentrations below those producing direct somatic death but this concept of sub-lethal effects is somewhat ambiguous. Indeed, in a real ecological context, some sub-lethal effects (including behavioral effects) will lead to lethal consequences since daphnia survival is

dependent on competition with other species, predation avoidance, search for food and avoidance of other multiple stressors (Newman and Unger 2003). For instance, by increasing their average speed, organisms raise their risk intake, making them more noticeable to predators. Decrease of the average speed may also adversely impact daphnia's survival since organisms eat less, are less able to flee from dangers and are then also more easily catch by predators (Reynaldi et al. 2011). On the other hand, behavioral effects such as avoidance may be considered as a benefit behavioral response: the increase of the swimming speed allows the exposed organisms to avoid the contaminated area of the habitat or a predator. Avoidance has already been reported in the literature for crustacean (De Lange et al. 2006, Eriksson Wiklund et al. 2006, Hellou 2011, Kravitz et al. 1999). A decrease of the swimming speed may also reduce energy cost, which is reallocated for detoxification mechanisms. On the contrary, an increase of the swimming speed leading to an overconsumption of energy may reduce energy allocation for less vital functions such as reproduction or growth etc. However, "beneficial effects" (hormesis) on one endpoint (i.e. the swimming speed), may not necessarily be beneficial for the overall fitness of the organism (Forbes 2000). Besides, we must keep in mind that even positive effects could be observed in certain tolerant species, chemical exposure may also negatively impair other more sensitive species and then disturb the ecological balance of ecosystems. As a keystone species, effects on *Daphnia magna* may lead to indirect effects on other organisms by impacting, for instance, the lower levels as a prey or the higher levels as a predator.

3.4 Biomonitors in aquatic biomonitoring

Various biotests have been developed using long-term and automatic observation studies based on the ability of the aquatic organisms to continuously sense a wide range of pollutants (Bae and Park 2014). Most often, behavior is measured in laboratory but some *in situ* systems measure behavior alterations directly on the field. Today, recent technological advances in computer hardware and software for image analysis systems led to several real-time biomonitor systems developments to detect behavioral changes in vertebrates and invertebrates. Due to the global concern about the impact of xenobiotic on the ecosystem and human health, a growing number of behavioral biomonitors has been utilized in biomonitoring programs as alternative or in complement to typical chemical monitoring.

Commercially available biomonitor systems using sensitive aquatic organisms from different taxa: bacteria, algae, water flea, mussel and fish (Osbild et al. 1995) have been applied *in situ* in Europe, US or Asia. Except on-line fluorescence monitoring system depending on the fluorescence change of luminescent bacteria, the rest of biomonitoring systems are based on swimming responses of sensitive aquatic organisms. These systems offer a continuous control of water quality and alarm can be triggered when significant behavioral changes occur. *Chlorella vulgaris, Daphnia magna*, *Japanese Medaka* and *Danio rerio* are most often used in biomonitors since these are standard species

typically used in risk assessment. Among these systems, organisms respond to various ranges of pollutants or toxic conditions. However, each species responds differently to a same pollutant and is specific to a certain application. For instance, biomonitors using bacteria (measuring the respiration, the bacterial growth or bioluminescence) are useful for wastewater treatment processes (Gerhardt et al. 2006) while algae biomonitors which record cell growth, oxygen production or fluorescence are a more specific tool for herbicides detection in water (Pandard et al. 1993, Rodriguez Jr et al. 2002). Bivalves are used in off-line (e.g. study of bioaccumulation of *in situ* caged bivalves) as well as on-line with shell closure recording on Dreissena-Monitor® (Kramer and Foekema 2001) but vibrations, temperatures, diurnal variations factors disturbs analysis and make its use difficult. Among the first used biomonitors, fish biomonitors was firstly focused on rheotaxis and avoidance reaction of polluted waves. Because of limit of sensitivity and ethical issues, this approach is now replaced by on-line tracking of fish trajectories for the determination of swimming speed, turning rate and swarm formation parameters. Several species can also be tested in the meantime (e.g. epibenthic and in benthic insects, crustaceans, and oligochaetes) with the Multispecies Freshwater Biomonitor® (MFB, LimCo International, Ibbenbueren, Germany) (Kirkpatrick et al. 2006).

3.5 On-line biomonitors for real time measurements

There is a special interest for the use of on-line biomonitoring systems downstream effluents and along rivers to detect toxic pollution peaks (Gerhardt et al. 2006). On-line biomonitoring systems, so-called Biological Early Warning Systems BEWSs, measure in real time continuous information on behavioral stress responses of caged test organisms exposed to *in situ* medium ideally provided by continuous flow. When a change in the behavior is observed, an alarm is triggered. The continuous information allows the detection of pollution peak and the effect onset in real time. Those systems can be directly used on the field, organisms are exposed in their natural environment and this is more representative of the realistic environmental pollution contrary to standard tests, which involve artificial environment. On-line biomonitoring systems are very useful in case of accidental chemical discharges by readily detecting dangerous lethal concentrations but also sublethal concentrations. Several on-line behavioral biomonitors are being used as early warning system throughout Europe to detect potential toxicity of water bodies using algae, Daphnia, mussels and fish toximeter (Hendriks and Stouten 1993).

Nevertheless, ecotoxicological relevance of the utilization of on-line biomonitors depends of the studied species. If algae are more specific for the determination of herbicides and mussels for metals, *Daphnia magna* is sensitive to an extended type of contaminants. For on-line biomonitors systems, *Daphnia magna* is suitable for short term pollution monitoring at concentration levels close to water quality standards and *Daphnia magna* tends to be more sensitive than *Japanese medaka,* this latter being more suitable for the long-term monitoring of accidental discharges (Ren and Wang 2010).

Beside, by comparing database of acute toxicity, *Daphnia magna* is also more sensitive than *Danio rerio* (Martins et al. 2007). Thus, we would like to investigate if behavioral responses are also more sensitive for sublethal concentrations of toxicants. Since *Daphnia magna* tends to be sensitive to a large range of substances compared to algae and more *sensitive* than fish, we can expect that *Daphnia magna* is a reliable species for behavioral assessment.

3.6 Behavioral analysis systems for *Daphnia magna*

The Bbe® Daphnia toximeter is successfully used as early biological warning system BEWS in water throughout Europe for the last 15 years. Alarm thresholds based on behavior changes from normal behavior have been established in toximeters (including Daphnia toximeter) for numerous compounds (Lechelt 2006) and serve as reference for internal use by operators (Lechelt et al. 2000). The Bbe® Daphnia toximeter has also been shown to be sensitive to a wide range of chemical warfare agents and their hydrolysis products (Green et al. 2003), and also to the non-calorie sweetener sucralose (Wiklund et al. 2012). This system, placed in derivation of the natural medium, measures in continuous physiological parameter (e.g. size of organisms) and several ethological parameters of daphnids (e.g. average speed, speed distribution, velocity class, height of swimming, distance between daphnia, active number of daphnia, fractal dimension and toxic index) during one whole week without maintenance needs. Ten daphnids neonates are generally placed in a measuring cell under flow-through condition. The medium is filtrated and regulated in temperature to 20°C. Daphnids are fed by periodically adding to the algae (*Chlorella vulgaris*) from a continuously operating fermenter. The system is designed to detect a deviation from a previous recorded behavior considered as "normal of reference" behavior. Each change in behavioral parameters is scored in a "toxic index" with a certain weight in function of the behavioral parameter relevance. When the "toxic index" overpassing a predefined fix threshold, an alarm is triggered and several water samplings are performed for further chemical analysis. However, it is currently unclear how high are these effect thresholds compared to classical ecotoxicological endpoints. The direct comparison of these thresholds to literature data is hampered by the large variability of literature toxicity data due to different sensitivities of daphnia clones, variations in exposure conditions or inter-laboratory differences (Baird et al. 1991). Furthermore, biological variability in behavior and differences in experimental conditions (salinity, pH, time) prevent direct comparisons of all the data gathered from Bbe® Daphnia Toximeter. Testing a large number of compounds in different concentrations and in replicates is not possible due to its limited test capacity.

A variety of behavioral analysis systems using daphnia under static conditions have already been developed but most of these systems focus on individual tracking and the few existing multi-tracking system of group of organisms systems offer a limited capacity testing. Real time multi-tracking system was firstly initialized by Wolf et al. (1998) with a system able to track 30 daphnids

moving simultaneously and led to the detection of sublethal concentration of cadmium. Then, a system able to follow 5 groups of 10 organisms moving simultaneously was developed by Untersteiner et al. (2003) under static conditions and gave promising results with a sublethal copper exposure. The most recent real-time system for the simultaneous observation of organism's group was developed by Jeon et al. (2008) but image resolution was still poor (up to 640×480 pixels) and the testing capacity reach a maximum of 8 measuring cells which do not allow performing sufficient replicates for an appropriate statistical analysis of the biological variability of swimming behavior.

Unfortunately, behavioral endpoints are underutilized in risk assessment for several reasons: it is difficult to objectively score some behavior, generation of biased information currently happens and, considerable inter-individual variability can exist in behavioral data. However, these difficulties can be minimized by a careful design and execution of experiment. Another difficulty also remains in the extrapolation of accurate results from well controlled conditions in laboratory to behavioral responses in field situations (Newman and Unger 2003).

4. MODE OF ACTION APPROACH IN ECOTOXICOLOGICAL RISK ASSESSMENT

4.1 Mode of Action information in risk assessment

4.1.1 Continuous progress in testing strategies for Ecological Risk Assessment (ERA)

To meet the objectives of environmental protection fixed by the new regulations (i.e., REACH or WFD), efficient ecotoxicological tests are required for the risk assessment of chemicals that may enter aquatic ecosystems and continuous improvement of testing strategies are recommended (Breitholtz et al. 2006). Facing the huge amount of chemicals potentially present in the environment, data collection procedure for toxicity determination may be time consuming and can lead to an overuse of acute toxicity assays and thus sacrifice of huge number of test animals. That is why, new strategies for experimental design and data interpretation are continuously needed in regulatory environmental assessment for the reduction of animal lethally testing and efficient use of finite laboratory and economic resources (ECETOC 2007).

Mode of action knowledge is important in classifying chemicals in function of their toxicity (Escher and Hermens 2002, Hutchinson 2002). As it represents an intermediate level of complexity in between molecular mechanisms and physiological effects, the mode of action is a simplified notion of toxicity, which may be very useful when the exact molecular target has not yet been identified or is subject to debate. Mode of action information has been reported as very useful as support for risk assessment (Clewell 2005) and could benefit to search for alternatives in ecotoxicology in general: the understanding of molecular, biochemical and cellular mechanisms may provide information on the potential effects and the time to observe effect onset and may also contribute to the performance of

mixture risk assessment across multiple chemicals (Barata et al. 2007, Neuwoehner et al. 2010, Syberg et al. 2008).

4.1.2 Contribution of mode of action approach for behavioral testing

If behavioral effects may greatly help for detecting toxicants in the water, standardization and field validation of these behavioral responses are still lacking. To date, no existing standardized guidelines are available for behavioral analysis. Furthermore, identification of the chemical that is behind the observed behavioral effects is still problematic. Numerous behavioral effects induced by chemicals are reported in literature, especially for fish and crustaceans. However, the shortcoming of the actual behavioral testing approach is that chemicals are either selected in random manner, either for a specific chemical assessment, which results in high heterogeneity in chemicals tested. Testing all pollutants potentially present in the environment is not realistic, a selection of test chemicals of specific concern or with respect to their mode of action may help prioritization (ECETOC 2007). For an explorative study of the sensitivity of our new behavioral system analysis, the selection of test chemicals with different mode of action is a good way to explore the different type of behavioral effects that can be observed. Besides, the selection of chemicals by their mode of action may potentially facilitate extrapolation of observed behavioral effects and time of effect onset of compounds with a similar mode of action. Indeed, in theory, compounds sharing the same mode of action should behave in a similar manner and should display similar toxic effects whose behavioral effects (Newman and Unger 2003).

4.2 Definition and classification of Mode of Action

4.2.1 Concept of Mode of Action

The toxicity of a substance depends on several factors:

- Concentration
- Time or frequency of exposure
- Toxicokinetic process
- Toxicodynamic process (i.e. Mode of Action)

While toxicokinetic represents the time for which a toxicant reach target sites (involving different processes such as absorption, distribution, biotransformation and excretion), toxicodynamic (i.e. Mode of Action) is linked to the internal concentration for which an effect is observable in the organism level and describes time effect onset, duration and recovery. According to Newman and Unger's paper in 2003, a mode of action is defined as all physiological and ethological changes that characterize a biological answer resulting from the exposure of a living organism to a substance. The mode of action must be distinguished from the mechanism of action: the latter refers to the understanding of the entire detailed sequence of toxicological events (i.e. at the molecular level) whereas mode of action, derived from this mechanistic evaluation, focuses on key events that lead to toxic effects (ECETOC 2007). In

other word, mode of action cannot be described as some distinct molecular mechanisms but rather as simple evidence of toxic effects.

4.2.2 Different classification schemes through literature

The principle of classification of chemical through mode of action is to find similarity in toxic effects in order to categorize biochemical mode of action in class and sub-group. Theoretically, compounds from the same mode of action should behave in a toxicologically similar manner and display similar toxic effects whose behavioral effects (Robinson 2009). So far, few information about Mode of Action are available but some classification schemes that allocates chemical in a proper Mode of Action class for different aquatic organisms have been reported in the literature. Initially based on a chemical class approach, QSARs methodology evolved to a more consistent approach based on toxic mode of action assumptions (Schultz et al., 1998). Indeed, it has been found more appropriate to combine chemicals by their toxicity mechanisms instead of chemical classes because compounds of the same chemical class can sometimes exhibit different toxicity mechanisms (Russom et al. 1997). Several studies on toxicity assessments or prediction using a mode of action approach are available in the literature (Ren 2002, Schirmer et al. 2008, Wenzel et al. 1997). In this present study, the modes of action assignments were evaluated and classified by gathering information mainly from QSARs studies but also from pharmaceutical/toxicological literature and supported with some toxicodynamics knowledge. Modes of action assignment are mainly established for organic compounds but the fundamental statement is meant to be generally applicable.

In 1992, Verhaar and his collaborators firstly initiate the concept of Mode of Action MoA categorization to predict the acute toxicity of organic compounds in fish (Verhaar et al. 1992). This classification is now well recognized and includes four classes: MoA1 inert chemicals with non-polar narcotic properties, MoA2 less inert chemicals through polar narcosis, MoA 3 reactive chemicals and MoA4 specially acting chemicals. Here, we propose an extended mode of action classification from gathered previous classification from literature.

- **The inert toxicity Class 1 (MoA1)** is formed by several substituted hydrocarbons with no specific functional groups, such alkanes, alcohols, ethers, aldehydes, ketones, esters and benzenes with halogen substituent. These narcotics compounds act through a non-specific manner on the cell membrane (Van Wezel and Opperhuizen, 1995) leading to the disruption of the normal cell membranes functioning. Their toxicity can be well predicted from their hydrophobicity of the substance by calculating an expected effect concentration, from the octanol/water partition coefficient (log K_{ow}). This inert toxicity

class is also called “baseline toxicity” considering the fact that any chemical will be at least as toxic as its log Kow indicates.

- **Less inert toxicity class 2 (MoA2)** is composed of polar substances with acid function like substituted phenols, nitrobenzenes, pyridines, anilines and aliphatic and aromatic amines. These polar narcotics, as non-polar narcotics, act non-specifically on the cell membranes. However, due to their tendency of forming hydrogen bonds and so binding to membranes, polar narcotics show slightly higher toxicity than the “baseline toxicity” predicted by log K_{ow} (Ramos et al. 1998). The distinction between non-polar and polar narcosis has been notwithstanding contested and challenged with another toxicity estimation of “general narcosis”, using the membrane-water partition coefficients, log K_{MW} instead of the log K_{OW} (Escher and Hermens 2002, Hodges et al. 2006). However, the toxicity difference between non-polar and polar narcosis has been further demonstrated and is based on a difference in the 3-D physical chemistry of the water-membrane partitioning process (Roberts and Cotello 2003). The distinction between polar and non-polar narcotics can be explain as the fact that polar narcotic molecules are associated with the head groups of the membrane phospholipids whereas non-polar narcotics molecules move freely in all direction in the membrane

- **Reactive chemicals class (MoA3)** can act through oxidative phosphorylation uncoupling (e.g. tetra and pentachlorophenols), Michael addition (e.g. α,β-unsatured carbonyls and quinines), Second order nucleophilic Substitution S_N2 (e.g. epoxides), aromatic nucleophilic substitution S_NAr (e.g. anilines), acylation (e.g. acyl halides and isocyanates) and Shiff base formation (e.g. aliphatic aldehydes) (Russom et al. 1997, Verhaar et al. 1992, Zhang et al. 2013). Reactive substances can react in a non-specific manner with certain chemical structures contained in biological molecules by forming irreversible covalent liaisons leading to membrane damage, protein damage and depletion or DNA damage (Escher and Hermens 2002). Toxicity of reactive chemicals is hence higher than chemicals belonging both to MoA1 and MoA2 classes.

- **Specifically acting chemical class (MoA4)** has been refined from previous classification and aimed to sub-structured specific mode of action class into several sub-classes based on target site. For example, neurotoxics, which are part of the same mode of action class, can be divided in many sub-classes since toxic mechanisms pathway is contrasted.

In (1997), Russom et al. proposed a different classification based on 8 classes of mode of action (i.e. 3 narcotics categories, oxidative phosphorylation uncouplers, respiratory inhibitors, electrophiles/proelectrophiles, acetylcholinesterase inhibitors and nervous system seizure agents). In 2003, Rang et al. also proposed to extend the MoA4 class into 4 pharmaceutical sub-classes based on protein receptors, enzymes, ion channels and transporters (Rang et al., 2003). But we decide to stay with the Verhaar's classification, which is well recognized and currently used. However, we must keep in mind that among this MoA-based classification scheme taken from Verhaar's literature, a moderate concordance has been shown in discriminating narcotics effects levels from excess toxicity. Beside, a poor prediction power and a non-negligible amount of substances cannot be classified in this Verhaar MoA classification scheme. Assignment of a chemical to a mode of action can be difficult and mistake can be done in mode of action allocations (von der Ohe et al. 2005). Furthermore, other factors such as uptake, metabolism and excretion of toxicants and experimental uncertainty can lead to miss-classification of mode of action category (Neuwoehner et al. 2010).

4.2.3 Introduction to excess toxicity concept

A toxic ratio T_e reflecting the excess toxicity from the "baseline toxicity" can be employed and calculated by dividing the predictive EC_{50} baseline toxicity from QSAR equation by experimental EC_{50} value obtained for chemical (1).

$$Te = \frac{EC_{50,baseline}}{EC_{50,\exp}} \qquad (1)$$

According to Verhaar (1992), the excess toxicity ratio T_e of less inert chemicals and the toxicity of reactive chemicals, as well as specifically acting compounds is spread from 10 to 10^4. The T_e value of 10 was taken to distinguish narcotics toxicity to excess toxicity. However, this threshold classification was derived using acute fish toxicity. A similar Toxic ratio TR has been reported in other studies with other thresholds for *Daphnia magna* (i.e., Baseline logTR<-1, Less Inert: -1<logTR<1 and Excess toxicity log TR>1) (von der Ohe et al. 2005, Zhang et al. 2013).

4.3 Application of mode of action classification for *Daphnia magna*

The aim of this work is to define a classification scheme of several distinctive classes and assign a particular mode of action to chemicals for *Daphnia magna* toxicity assessment. To well understand toxicodynamics process (i.e. Mode of Action), toxicokinetics elements are needed concerning toxicant or metabolite activation/detoxification processes. Several toxicokinetics studies for *Daphnia magna* are available on adsorption (Shitara et al. 2006), distribution,

metabolism/detoxification (Kashian, 2007) and excretion process for organothiophosphates (Kretschmann et al. 2011), metals (Guan and Wang, 2006, Deleebeeck et al., 2007) or PAHs (Gourlay et al. 2005).

Narcosis is a universal mode of action in all organisms and target tissues since basal cellular structures and functions are well-conserved throughout eukaryote evolution (Escher and Hermens 2002). Except for narcotics, the extrapolation of MOA assignment across trophic levels is in contrast limited (von der Ohe et al. 2005). Indeed, some toxic modes of action have been found to be species dependent (e.g. disturbance of photosynthesis for algae) (Escher and Hermens 2002, Zhang et al. 2013). According to Enoch et al. (2008), similar effects of reactive chemicals are expected for all species. For specific and multiple modes of action, categorization of mode of action should be driven according to the target site to predict toxic effect across different organisms and hitherto, it has poorly been reported in literature. The baseline toxicity characterization is species dependent (Zhang et al. 2013) so specific QSARs equation has to be taken for acute toxicity prediction in *Daphnia magna*. Many groups of pesticides act through a neurotoxic mechanism (mainly anticholinesterases insecticides including organophosphates and carbamates). Nevertheless, even among the same class of mode of action (e.g. neurotoxic), contrast in toxicity potency, time of effect onset and duration of effects may be observed. That is why, it is important to subdivide this neurotoxic category in several sub-classes for each complex brain pathway.

4.4 Selection of 12 testing substances

The majority of industrial chemicals act through narcosis (Enoch et al. 2008, Schirmer et al. 2008). Hence, narcotics dominate the list of tested compounds whereas specifically acting chemicals such as pesticides are known to be much more toxic than narcotics and must be taken into considerations. Despite substantial efforts of manufacturers to reduce their toxic impact and increase their degradability, pesticides continue to impact non-target organisms such as *Daphnia magna*. Consequently, our approach will focus to cover a range of modes of action as wide as possible with a specific concern on pesticides. For instance, on the 225 industrial chemicals dataset studied by Russom et al. (1997), 71% have been classified as narcotics, 20 % as reactive chemicals, 3% uncoupling agents, 2% respiratory inhibitors and 4 % in CNS agent seizure. Some chemicals are straightforward in their mode of action; others can involve a complex series of damages (i.e. multiple mode of action). To simplify the methodology, we aimed to select mode of action, which are clearly identified. Reactive chemicals (MoA class 3) have been intentionally removed from our testing chemical selection because of their high degradation potency over time, which may result in problems for keeping constant concentration and analysis and is not relevant for an exposure of 48 hours. Because Verhaar et al. (1992) did not include metals in his classification; we add the copper pentahydrate sulfate in the multiple class mode of action for our chemical selection.

- **Ethanol** and **isopropanol** have been selected as **non-polar narcotics**. These molecules have dipole moment but they act through non-polar narcosis (as depicted in the Figure 7). The inert and less inert toxicity is usually called non-polar and polar narcosis respectively. However, the polar characteristic does not interfere with the toxicity. Thus, instead non-polar/polar narcosis, inert/less inert term is more appropriate concerning chemical toxicity.

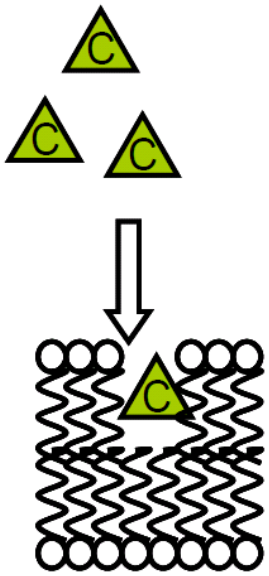

Figure 7: Principle of narcosis, chemicals "C" enter in the membrane cell and disturb its functioning.

- **Specific mode of actions:** neurotoxic may highly impair the behavior. That is why, we selected various sub-class among neurotoxic mode of action. The organophosphate **trichlorfon** and the carbamate **carbofuran,** which are two-acetylcholine **esterase inhibitors** were selected. The Acetylcholinesterase is in charge of the breakdown of the neurotransmitter acetylcholine into choline and acetic acid once transmission is done. Once the signal is transmitted, the acetylcholine must be breakdown to end the neuronal transmission (see Figure 8). The inhibition of the AchE leads to the accumulation of Acetylcholine and interfere with the normal functioning of the nervous system. Acetylcholinesterase enzyme appears to be widely conserved across the Ecdyzoa (ECETOC 2007). Organophosphates and carbamates are extensively used insecticides in agriculture since they exert high toxicity but are rapidly degraded into the environment. However, organophosphates lack target specificity and can cause adverse effects on non-target species such as *Daphnia magna*. Their release in the environment may alter the balance of daphnia community and other invertebrates. Within carbamates, carbofuran is a reversible acetylcholinesterase inhibitor (Barata et al. 2004). Hence, we may expect

recovery at non-lethal concentrations. Behavioral impairments have been previously correlated with AchE inhibition in fish (Sandhal et al., 2005).

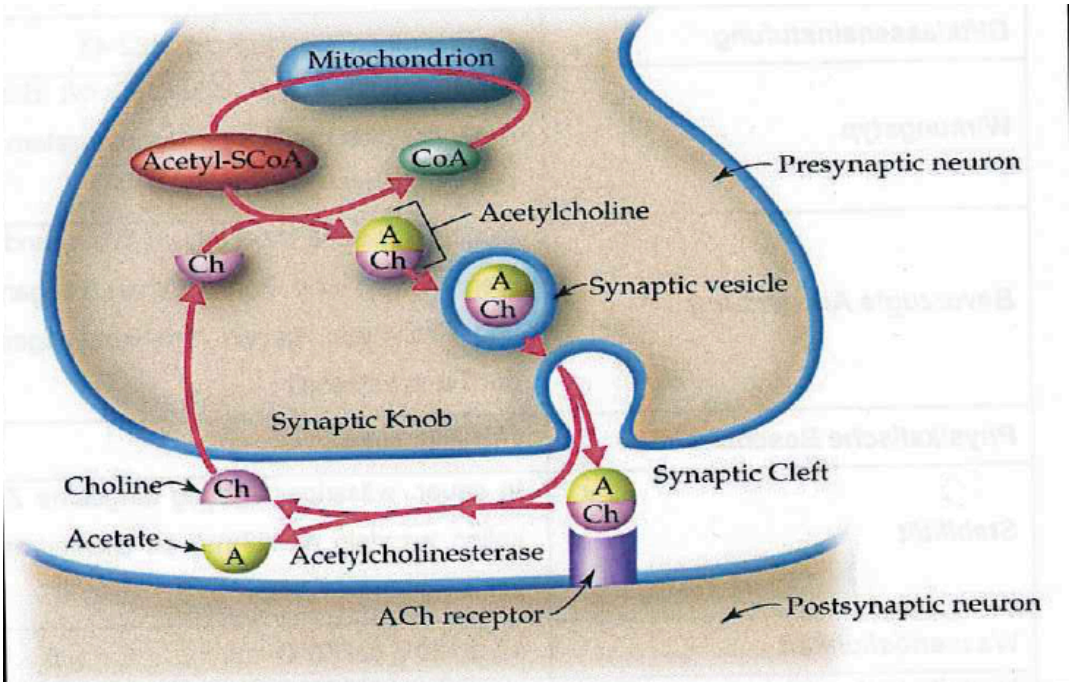

Figure 8: **role of the enzyme acetylcholinesterase in synaptic ends of cholinergic neurons**

- The pyrethroids **esfenvalerate** and **cypermethrin**, as all pyrethroids exhibit a common mode of action by **modulating sodium channels functioning.** The sodium pump (Na/K) is the active transport system responsible for maintenance of the gradient Na+ and K+ in the axonal membrane. Pyrethroids induce the reversible blocking of sodium channels in the neurons, leading to impaired action potential along the axon and hyper-excitation followed by muscle paralysis and death by respiratory (Hodges, 2004, Burr and Ray, 2004). Cypermethrin is a widely used insecticide for agricultural or domestic purposes. Effects on the swimming ability and feeding efficiency of *Daphnia magn*a after cypermethrin exposure have been already reported in the literature at concentrations occurring in freshwater ecosystems after pesticide applications (Christensen et al. 2005).

- **Fipronil** and **abamectin** are known to interact with **the glutamate-gated channels and GABA (γ-amino butyric acid)-gated chloride channels** in arthropods. Fipronil and abamectin are respectively antagonist and agonist of GABA receptor which result in a strong chloride influx leading to a disruption of continuous nervous transmission (Tisler and Erzen 2006). Organisms are hence over-stimulated and paralyzed before dying. *Daphnia magna* has previously found to be sensitive to abamectin but so far fipronil has not been studied.

- **Sertraline hypochloride is a psychotropic drug, which** has been selected as **selective serotonin reuptake inhibitor (SSRI).** Pharmaceuticals are becoming of recent concern since residues have been found in water and may potentially affect aquatic organisms. Among common pharmaceuticals mode of action, selective serotonin reuptake inhibitors are widely used for psychiatric disorders. Sertraline has been shown to alter the reproductive physiology of *Daphnia magna* (Constantine and Huggett 2010, Henry et al., 2004, Minagh et al., 2009) . It was shown to interact with carbohydrate metabolism and synthesis of ecdysteroids (Campos et al. 2012). This substance acts by blocking the reuptake of serotonin in the nerve synapses, leading to an increase of the effective serotonin concentration in the intrasynaptic space and the stimulation of serotoninergic neurons, as it is depicted in the figure 9 (Campos et al. 2012). Since different serotonin receptors have been found in decapod crustaceans, we may expect effect of sertraline on *Daphnia magna* (Spitzer et al., 2008).

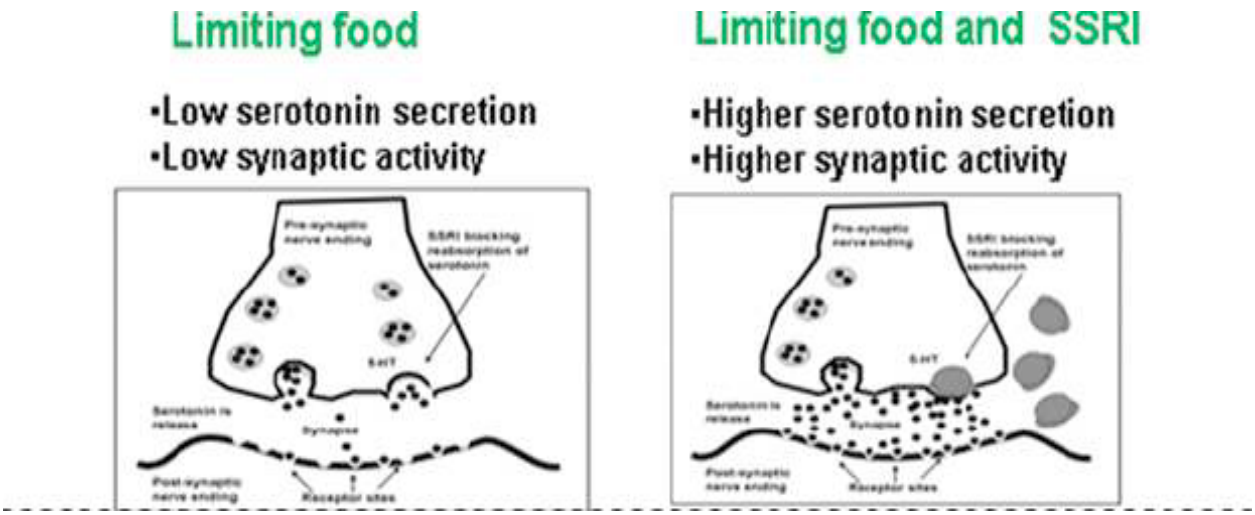

Figure 9: Selective serotonin reuptake inhibition process in (Campos et al. 2012).

- **Caffeine** is an **adenosine A1 and A2 receptor antagonist** involved in sleep regulation in the nervous system which may act by blocking the A2A receptor (Rihel and Schier 2013). Caffeine is frequently found in the aquatic environment at low concentrations (856 ng/L in France, (Bouissou-Schurtz et al. 2014)) and is constantly released in the environment via the food (80%), pharmaceutical cosmetics (16%) and technical applications (1%). Since, caffeine was reported to decrease sleep in zebrafish (Rihel et al. 2010), we expect to observe a similar behavioral effect on *Daphnia magna*.

- The neonicotinoid **imidacloprid** is an **agonist of the postsynaptic nicotinic acetylcholine receptors nAChR** (Matsuda et al. 2001). The mode of action is depicted in Figure 10. This insecticide is increasingly used to replace the more toxic insecticide diazinon and hence, imidacloprid is likely to be found in large quantity in the aquatic environment. Imidacloprid has been found to be persistent and not readily biodegradable in aquatic environment (Tišler et al. 2009). Even if imidacloprid has been found less toxic than diazinon, still acute and chronic effects were observed in *Daphnia magna* (Jemec et al. 2007, Tišler et al. 2009). Besides, behavioral effects (i.e. increase of the swimming speed) have been recently measured on the species *Daphnia pulex,* so we strongly attend to observe behavioral effects on *Daphnia magna* (Zein et al. 2013).

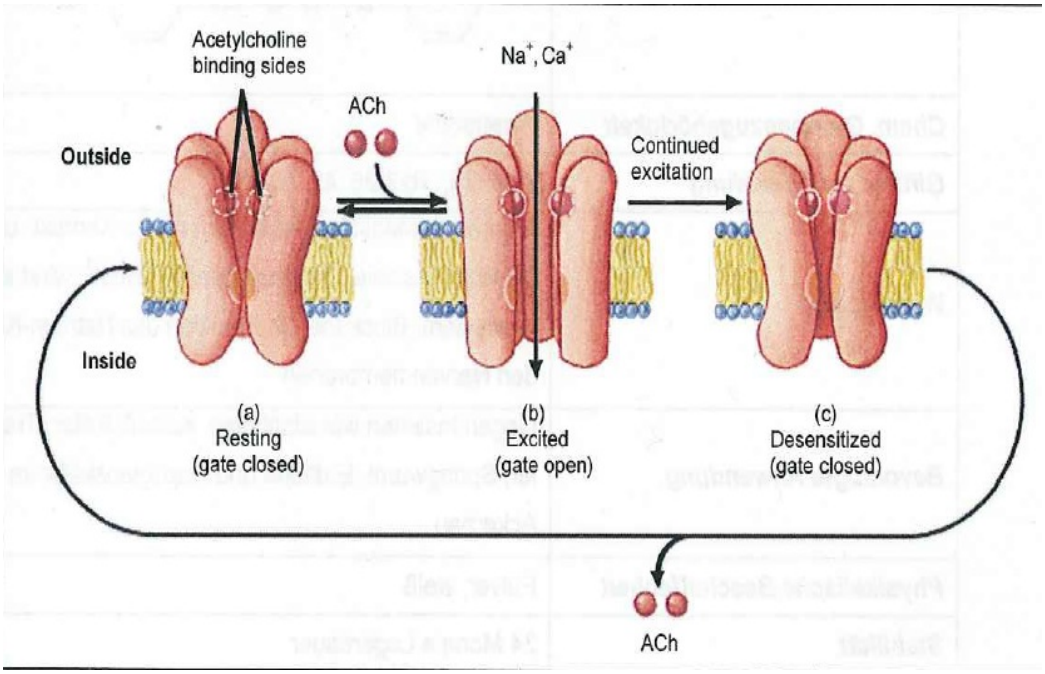

Figure 10: **mode of toxic action of agonist of the nicotinic acetylcholine receptors nAChR (Lewandowska, 2004).**

- As **multiple mode of action**, we selected the **sulfate copper pentahydrate.** This latter is an essential micronutrient which is incorporated in more than 30 different enzymes, whose the extracellular hemoglobin involved in the oxygen transport (Barata et al. 2005, Untersteiner et al. 2003). However, its toxicity involves more complex mechanisms (e.g. oxidative stress) not only attributed to systemic toxicity but rather to the detoxification mechanisms for the osmoregulation of copper in the organism. Copper is a component of various pesticides and is hence widely released in aquatic environment. Daphnia is known to be acutely sensitive to heavy metals whose copper (Arambasic et al., 1995). Chronic effects on the reproduction have been also documented (Sobral et al. 2001). Besides, behavioral effects have also been observed on *Daphnia magna* (Jeon et al. 2008, Untersteiner et al. 2003).

CHAPTER II. DEVELOPMENT OF A NEW MULTI-CELL EXPOSURE SYSTEM FOR CONTINUOUS TRACKING OF DAPHNIA BEHAVIOR FOR TOXICITY ASSESSMENT

1. DEVELOPMENT OF A NEW DAPHNIA BEHAVIORAL ANALYSIS SYSTEM

1.1 Conception of the new daphnia behavioral system with high-test capacity

For a systematic assessment of the sensitivity of behavioral endpoints in daphnia, a system that allows the behavioral analysis of daphnids groups with different concentrations and sufficient replicates is required. In addition, for a hypothetic comparison of behavioral endpoints with the standard immobilization endpoint, experimental condition, i.e., time of exposure, light, temperature, feeding, etc., should be similar or closed in both tests.

We developed together with the Viewpoint company (Life® Technologies, France), a new multi-cell exposure system named "Multi-DaphTrack" for the simultaneous video tracking and behavior analysis of a large number of groups of daphnids for several hours or days. First of all, a requirement document has been established with the following specifications needed for the experimental purposes:

- Experimental platform with numerous cells of 20 mL (>18 cells)
- Controlled exposure condition (stable temperature, illumination)
- 2-D front view video analysis (better suitable for daphnia swimming behavior)
- Tracking of very small juveniles daphnia (<24 hours)
- Multi-tracking of several groups of 10 daphnids
- Continuous tracking over 48 hours

Finally, we customized the ZebraBox® in order to record the behavior of 10 daphnids dispatched in up to 20 cells placed in a multi-cell platform with a camera (front view) and the software Zebralab® (Life® Technologies, France), see Figure 11.

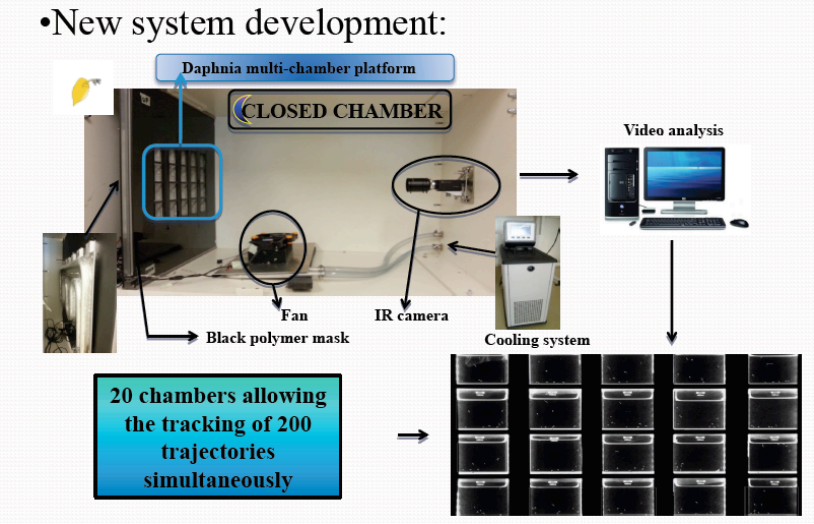

Figure 11: Conception of the new Daphnia behavioral analysis system with a high-test capacity

1.2 Experimental conditions set up

1.2.1 Feeding

The daphnia average speed is closely correlated with the size and age of organism (Dodson et al. 1997). As depicted in the Figure 12 of the preliminary report of INERIS (Chancerelle et al. 2010), when daphnids grow up, the average speed increase.

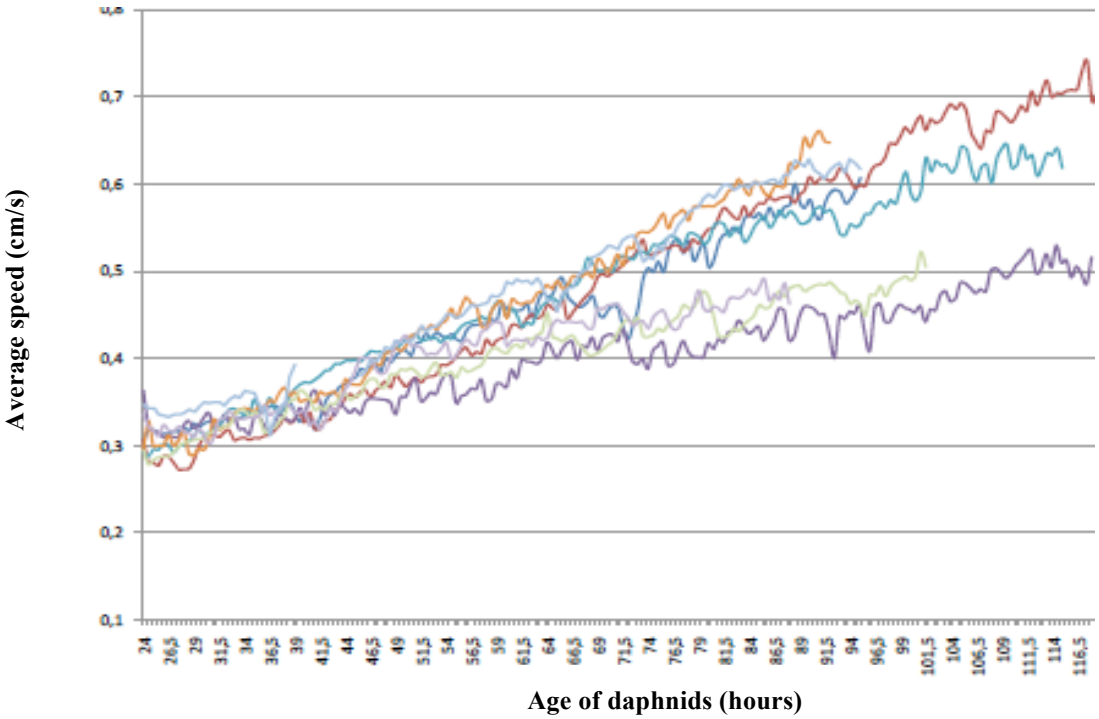

Figure 12: Average speeds of daily fed group of 10 daphnids exposed in the DaphToxI® under control condition (Chancerelle et al. 2010).

Furthermore, in molting period, the average speed may suddenly increase (see 13). Daphnids are not fed in the standard immobilization test. That is why, to compare with the standard test condition and to limit the growth of daphnia over time, we decided to not feed the daphnids during the whole experiment.

13: Increased average size and decreased average speed phenomena during the molting period of a single daphnia exposed to control condition in the DaphToxI® (Chancerelle et al. 2010).

1.2.2 Illumination

Infrared illumination was used throughout the experiment since it is invisible to daphnids and does not influence their behavior. The initial 50 cm x 50 cm infrared background illumination system placed beside the measuring cell was not good enough for efficient detection by the camera. Infrared Light-Emitting Diodes (LEDs) were subsequently placed around the experimental platform, which did improve daphnia neonate detection, but not enough. Infrared LEDs were finally placed in each vertical row to increase the light intensity on each side of the measuring cells, ensure the homogeneity of the light and thus achieve optimal detection. Nevertheless, light reflection on the optic glass exposure cell increased the noise of the final measurement and induced artefacts. To counteract this light reflection, black adhesive strips were adjusted on each optic glass cell wall to reduce light diffusion and reflection. Then, to avoid artefacts, a background refresh option was performed frequently as a noise filtering operation. The optimal frequency for refreshing the background was found to be every 60s. Threshold detection parameters (grey value for the contrast and pixel for size delimitation) were also set up for each exposure cell independently.

2. BASAL SWIMMING ACTIVITY OF DAPHNIA

2.1 Individual behavior in the "Multi-DaphTrack"

To study the basal swimming activity of *Daphnia magna* exposed to control conditions in the "Multi-DaphTrack", the individual swimming activity of single individuals was firstly determined (1 daphnia per cell, 14 replicates). The result of the individual average speed was homogeneous over time and comprised between a minimum of 1.4 and a maximum of 1.9 mm/s (Figure 14). The 48 h averaged swimming speed was equal to 1.7 ± 0.14 mm/s. This individual average speed values can be compared to values of the literature, e.g., in Dodson et al., (1997), an individual average speed was

found to be 8.04 ± 0.86 mm/s. However, in this study, Daphnids were older and consequently bigger (> 1mm instead of 0.2-0.6 mm in our study) and the size of the exposure chamber was also bigger (6400L compared to 20 mL in our study). To measure the rest frequency over the 48-h exposure, a high-resolution analysis was also performed with a high frequency time measurements and a long time frequency of background-refresh (30 minutes), enough to avoid artifacts and to keep daphnia tracking when they are at rest. Overall, given these results, **no marked biological cycle** was observed in individual daphnids over the 48 h exposure.

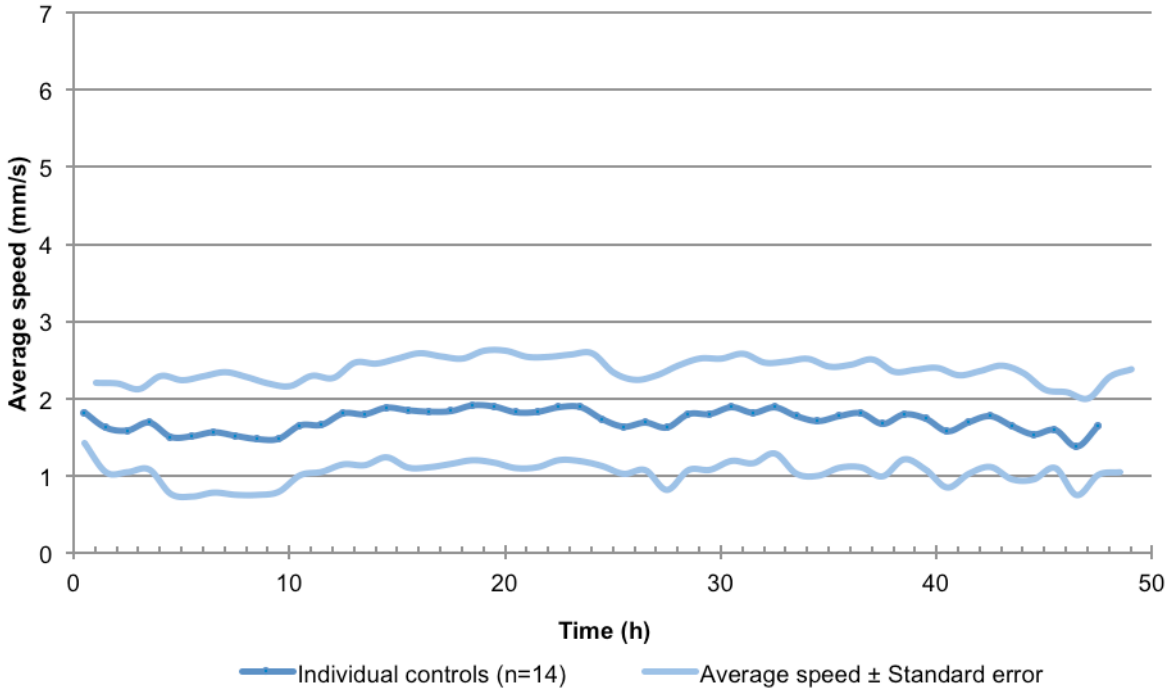

Figure 14: Individual average speed of *Daphnia magna* exposed to control condition over 48 h in the "Multi-DaphTrack".

2.2 Basal swimming activity of group of Daphnia

Under static conditions (i.e., in the "Multi-DaphTrack"), the average speed of group of daphnia was significantly higher than individual average speed. This difference may be related to the stimulation of organisms by interaction between individuals. As depicted in the **article I**; the average speed of 10-Daphnia group exposed to control condition in the "Multi-DaphTrack" was stable over time with a 48 h-mean swimming speed equal to 2.55 ± 0.11 mm/s. However, a slight decrease in the swimming speed was observed at the end of the experiment (down to 2.24 mm/s). Various numbers of organisms (3, 5, 7, 10, and 15) were also tested under control condition in triplicate to estimate the impact of daphnia density on swimming speed. In results, no significant differences were observed between the mean average speeds of a given group (averaged at 2.31 ±0.06 mm/s). Therefore density does not appear to stimulate daphnia average speed (expect for isolated individuals).

The average speed of 10-Daphnia group exposed to control condition in the flow-through DaphToxI® system, was significantly higher than in static condition (i.e., the "Multi-DaphTrack"). An average speed of 3.65±0.13 mm/s was observed for control conditions in the DaphToxI® system over a period of 48 h; daphnids swam 1.5 times faster in the DaphToxI® system than in the "Multi-DaphTrack" (2.55 ± 0.11 mm/s), which is most likely because of the stimulation of the imposed water flow in the flow-through system. The swimming speed slightly decreased over time in the "Multi-DaphTrack", whereas the average speed remained stable in the DaphToxI® system. The variability of the average speed per hour was, on average, equal to 0.10 mm/s in the "Multi-DaphTrack". The variability of the swimming speed per hour was much higher in the DaphToxI® (on average equal to 0.24 mm/s), which is logical given the lower number of replicate (65 in the "Multi-DaphTrack"/12 in the DaphToxI®. The variability is higher than in static or static-renewal tests and obviously due to additional sources of variability.

The number of active organisms was less uniform between replicates compared to the swimming speed parameter, and the standard error increased at the end of exposure. The swimming speed remains stable while the number of active organisms tends to decrease between the 20th and the 35th hours; thereafter the swimming speed also decreased. The decrease in both parameters can be explained by the exposure conditions: static conditions do not stimulate daphnia movement; the neonates were not fed throughout the whole experiment and likely became tired and need to stay at rest.

The measurement of path angle in daphnia exposed to control condition in the "Multi-DaphTrack" provides less clear results. Approximately 12 % of daphnia swam straight (0 to 2°), 20 % of daphnia swam with a very slight path angle (2 to 4°), 20% change their path angle with 4 to 8°, 18 % of daphnia swam with a bigger path angle (8 to 20°) and 16% with a high path angle (20-45°). Finally, 7% of daphnia swam with a very high path angle (45 to 90°) and 5% only turn with an angle above 90°). However, the angle class may need to be refined and, given the geometry of the measuring cell (depth 1 cm), the reliability of this parameter is uncertain in this experimental condition.

The position of neonates in the measuring cell was not recorded during experiment. However, by visual observation, we can globally say that daphnia swim homogeneously through the water column. No diel vertical migration process was observed. This observation concurs with the fact that experiments were conducted in darkness with only infrared light, which is not visible for daphnia, and that diel vertical migration is governed by phototaxis (Ringelberg 1964). At the end of exposure, daphnids swam near the bottom, but this is also correlated with the observed decrease in activity. Overall, the average speed parameter of daphnia's group exposed to control condition in both systems was more homogeneous over the entire 48 h exposure compared to the number of active organism's parameter. Furthermore, the path angle change parameter is difficult to exploit. That is why we decide to mainly focus on the average speed parameter for toxicity measurement.

3. EXPERIMENTAL METHODOLOGY FOR BEHAVIORAL TESTS

3.1 Experimental methodology of chemical exposure in the "Multi-DaphTrack" system

To define the concentration range for behavioral tests, acute *Daphnia magna* toxicity tests were first performed according to the OECD 202 test guideline (OECD 2004) in order to select 5 nominal concentrations covering effect concentrations below EC_5 (48H) up to EC_{100} (48H) of the acute test for the test in the "Multi-DaphTrack" system (see Figure 15).

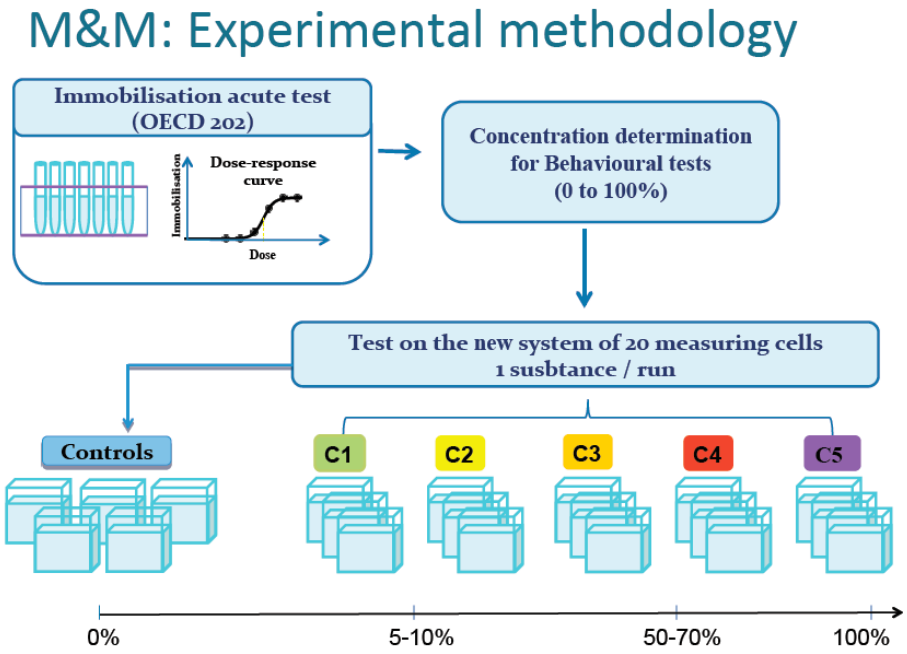

Figure 15: Experimental methodology for chemical exposure in the "Multi-DaphTrack" system.

3 replicates were tested for each exposure treatment and 5 replicates for the controls. When methanol is used as solvent for the chemical exposure, 2 replicates of ISO water and 3 replicates of ISO water with 0.01% of methanol were tested for the controls. Each exposure cell was filled with 20 mL of the test solution, sealed with PARAFILM "M" ® and kept at 20 ± 0.5°C. 10 neonates were carefully and randomly placed in each test exposure cell. In addition to 48-hour video tracking, immobilization of daphnia was determined at the end of the 48-hour test.

3.2 Experimental methodology for chemical exposure in the DaphToxI® system

To compare behavioral trends from the "Multi-DaphTrack" system to a commercialized BEWS currently used in biomonitoring programs, one control and two replicates of a similar concentration tested in the "Multi-DaphTrack" system were tested in parallel for chemical exposure in the DaphToxI® system (Bbe® Moldaenke, Kiel, Germany) (see Figure 16). Ten neonates were placed in each of the 3 exposure cells (25 mL) in different DaphToxI® systems. The experiment was performed under flow-through conditions at 33 mL/min and 20 ± 1°C, in which daphnids were first

acclimated with an ISO water solution for 2 hours. The analysis of the behavior started at the beginning of the chemical exposure by replacing the ISO water with the test solution in a closed circuit after complete ISO water evacuation (the time was previously estimated at 3 minutes by a colorimetric measurement). To be consistent between the immobilization test and the test in the "Multi-DaphTrack" system, the experiments in the DaphToxI® were conducted without a food supply.

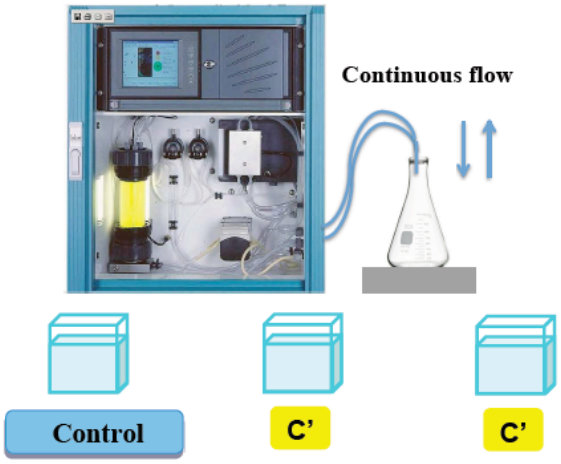

Figure 16: Experimental methodology for chemical exposure in the DaphToxI® system.

ARTICLE 1 - A NEW MULTI-CELL EXPOSURE SYSTEM FOR CONTINUOUS TRACKING OF DAPHNIA BEHAVIOUR FOR TOXICITY ASSESSMENT

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ABSTRACT: For several years, video tracking systems have been developed to analyze alterations in daphnia swimming and provided early signal of chemical stress. However, these systems offer a limited test capacity that does not allow for a systematic analysis of the robustness of behavioral endpoints. Hence, with the recent advances in behavior tracking technology, we developed a new behavioral analysis multi-cell exposure system named "Multi-DaphTrack" with a high throughput testing capacity for assessing the behavioral response of *Daphnia magna*. The insecticide esfenvalerate was tested on daphnid neonates at several concentrations for 48 hours in order to (i) evaluate the performance of this new system and (ii) compare the sensitivity of our new multi-cell system with standard immobilization assay and the Bbe® Daphnia toximeter. Overall, the results demonstrated that our new "Multi-DaphTrack" system can detect significant behavioral effects of esfenvalerate at concentrations as low as 0.14 µg/L from a minimum of one hour of exposure. Similar rapid behavioral effect trends were observed in Bbe® Daphnia toximeter. Behavior endpoints proved to be more sensitive compared to the standard immobilization acute toxicity endpoint. Significant behavioral changes were observed at esfenvalerate concentrations occurring in contaminated rivers from agricultural areas in Europe and North America. According to our results, the "Multi-DaphTrack" system represents a powerful and convenient tool for the assessment of chemical toxicity and water quality.

KEYWORDS: *Daphnia magna*, behavioral analysis system, water quality, biomonitoring, risk assessment

INTRODUCTION

When assessing the risk of chemicals released into the environment, potential toxicity is usually assessed using standardized toxicity tests on representative organisms. The immobilization test on *Daphnia magna* is one of the most frequently used standardized tests (ISO 6341 2012, OECD 2004) for assessing the hazardousness of chemicals and also for monitoring water quality. Indeed, the test is simple, fast and cost effective, and *Daphnia magna* is highly sensitive to a wide range of chemicals (Martins et al. 2007) and is representative of freshwater organisms (Baird and Van den Brink 2007, Mark and Solbé 1998). However, in many surface water or effluent toxicity assessments, *Daphnia magna* immobilization is not sensitive enough to measure water quality since sub-lethal effects can occur at much lower concentrations. Several indications of chemical stress produced by pollutants on organisms are ignored in the standard acute toxicity test, since this test only focus on immobilization at two pre-defined exposure times (i.e., 24 and 48 hours). Instead of immobilization, the reduction in the organisms' overall state of health can provide useful information on induced adverse effects and allow a toxicity threshold to be determined. Significant impacts (e.g., altered behavior, growth and reproduction) can occur below the median effective concentration (EC_{50}) level (Dodson and Hanazato 1995). Furthermore, it is not common practice to monitor effects over time despite the fact that it is well known that the effects of chemical stressors change temporally and depend on exposure duration (Heckmann et al. 2010). Detection of sub-lethal effects over time may therefore be of great assistance in water quality assessment.

To protect the aquatic ecosystem, a rapid assessment of the toxic potency of environmental pollutants is needed. Behavior is a sensitive indicator of acute/sub-lethal toxicity (Coelho et al. 2011), which is especially suited to detect stress induced by realistic environmental contaminant exposure concentrations. Therefore, behavioral monitoring of encaged organism is increasingly used for water quality assessment as an alternative to, or supplement to chemical monitoring (Bae and Park 2014). Furthermore, behavioral tests provide early and intermediary responses prior to the death of organisms since toxic stress can induce rapid behavioral changes in exposed organisms (Amiard-Triquet and Amiard 2013). Significant alterations of daphnia swimming parameters have already been reported after exposure to xenobiotics metals (Jeon et al. 2008, Untersteiner et al. 2003), pesticides (Zein et al. 2013), nanoparticles (Artells et al. 2013)), surface water samples and cyanotoxins (Ferrao-Filho et al. 2014). These alterations can impact the overall state of health and survival of organisms, which may lead to long-term changes at population and community levels (Duquesne and Küster 2010). Indeed, if swimming speed increases, the energy used for normal metabolic functions (e.g. growth, reproduction and locomotion) can be reallocated to locomotion and may therefore impact the fitness or the long-term survival of organisms (Knops et al. 2001). Moreover, adaptive behavior such as avoidance or alteration of motility under toxic stress can impact decision-making (e.g. location), making organisms more noticeable to predators.

There are currently two different types of system that monitor daphnia behavior: the first type, dedicated to water monitoring, provides real-time signal processing, while the second type is designed to better characterize toxic effects on swimming behavior. Systems dedicated to water monitoring often have a limited number of measuring cells with a flow-through system and automated image analysis algorithms to provide real-time signal processing. The chief aim of these systems is to trigger alarms when abnormal behavior is observed, which then can initiate water sampling or the shutdown of the water works inflow. The current behavioral analysis systems available on the market using daphnia under flow-through conditions (LimCo®, Bbe®) are used to detect toxic pollution peaks in effluents or rivers (Gerhardt et al. 2006). The Bbe® Daphnia toximeter, for instance, has been successfully used as an early warning system for chemical pollution in surface river water throughout Northern Europe for the last 15 years.

The other type of system designed to monitor the toxic effects of chemicals on daphnia behavior is generally developed in laboratories and mostly under static conditions. These laboratories systems monitor one or several swimming parameters such mean velocity (most frequently used), activity/rest, distance travelled and path angle changes. Compared to commercialized *in situ* systems, laboratories systems are more or less hand-crafted systems developed to monitor the behavior of many daphnids simultaneously and often combine a single or several cameras with cheap tracking software. So far, the system with the highest test capacity for simultaneously observing a group of organisms was developed by Untersteiner et al. (2003), but the testing capacity reached a maximum of 6 measuring cells. Other systems have been developed more recently for behavior analysis on individual/small groups of daphnia. For instance, system capable of individually monitoring 6 *Daphnia magna* moving simultaneously under static conditions was developed by Jeon et al. (Jeon et al. 2008). To date, recent technological advances and enhanced detection resolution and computer performance have increased the testing capacity tracking to up to 12 replicates of group' organisms (Artells et al. 2013) and resulted in the high throughput screening assay of individuals with 24 replicates (Zein et al. 2013). So far, most of these systems focus on tracking individuals or small groups of organisms, effects are often observed over a limited time (e.g., a single pre-defined time or a few hours) and the experimental conditions do not follow the standard acute toxicity test protocol.

The behavioral analysis systems currently available on the market offer a limited test capacity that does not allow for a systematic analysis of the robustness of behavioral endpoints. The aim of the study is (i) to improve behavioral analysis by developing and validate a new multi-cell exposure system with an optimized test capacity, (ii) to establish the link between daphnia behavioral responses and exposure to a well-known neurotoxic insecticide, and (iii) to compare the sensitivity of the behavior effects detected in our new system with the standard immobilization endpoint and a current online biomonitoring system, i.e. the Bbe® Daphnia toximeter. To this end, we developed together with Viewpoint company (Life® Technologies, France), a new multi-cell exposure system named

"Multi-DaphTrack" for the simultaneous video tracking and behavior analysis of a large number of group of 10 daphnids dispatched in up to 20 cells for several hours or days. To compare the behavioral endpoints to the standard immobilization endpoint, the "Multi-DaphTrack" system was adapted from standard acute toxicity test conditions. The neurotoxic insecticide esfenvalerate was chosen as model molecule inducing rapid behavioral effects as it is reported for crustacean. The neurotoxic insecticide esfenvalerate tested firstly in the "Multi-DaphTrack" system to validate this new behavioral monitoring system and secondly on the Bbe® Daphnia toximeter to compare the behavior trends of these two behavioral analysis systems. All experiments were conducted over a period of 48 hours at 20°C to allow a direct comparison with the results of the standard acute toxicity test.

MATERIALS AND METHODS

Design of the new behavioral multi-cell exposure system

The "Multi-DaphTrack" system was designed to simultaneously monitor a maximum number of groups of daphnids (group of 10 individuals) and to be directly compared to the acute immobilization tests. A test capacity of up to 20 replicates (5 concentrations in triplicate and 5 control replicates) was considered the best option between the high-test capacity and detection efficiency. 20 optical glass cells (50 x 50 x 10 mm) supplied by Hellma® were used as exposure cells and assembled in a vertical 4x5 rack. Infrared light-emitting diode (LED) strips with a wavelength of 850 nm were placed between each of the rack's vertical cell rows to ensure homogeneous cell illumination. After inserting the exposure cells, the rack was covered with an opaque polymer mask, masking the light sources and exposure cell walls to limit diffusive light and reflections. This multi-cell exposure platform was placed inside a 100 x 60 x 60 cm black box to exclude external illumination. A video of the experiment was recorded by an infrared digital HD camera with a high-resolution of 1600 x 1200 pixels operating at 25 frames/s and positioned squarely 54 cm from the rack containing the exposure cells. The Figure 17 shows the synthetic scheme of the system. Animals reflect infrared and were therefore detected as white silhouettes against a black background. The video was transferred directly to a computer and saved in AVI format. Raw data on daphnids positions x, y were extracted from the video with high time resolution using Zebralab® software algorithms (Viewpoint® Life Technology, France). Trajectories were reconstructed and several behavioral parameters were calculated for each measuring cell (each group of 10 daphnids): swimming speed, number of active organisms and change in path angle. Temporary averaged parameters for each daphnia group were then integrated into a time sequence of 1s (25 images) and averaged parameters were finally calculated and recorded on a predefined time bin (here=30s) over 48 hours and saved in an Excel file.

Simultaneous video tracking of 200 daphnia is challenging so many tests were carried out under control conditions to optimize the detection rate. Various numbers of organisms per cell (1, 3, 5,

7, 10, and 15) were also tested under control conditions in triplicate to estimate the impact of daphnia density on swimming speed.

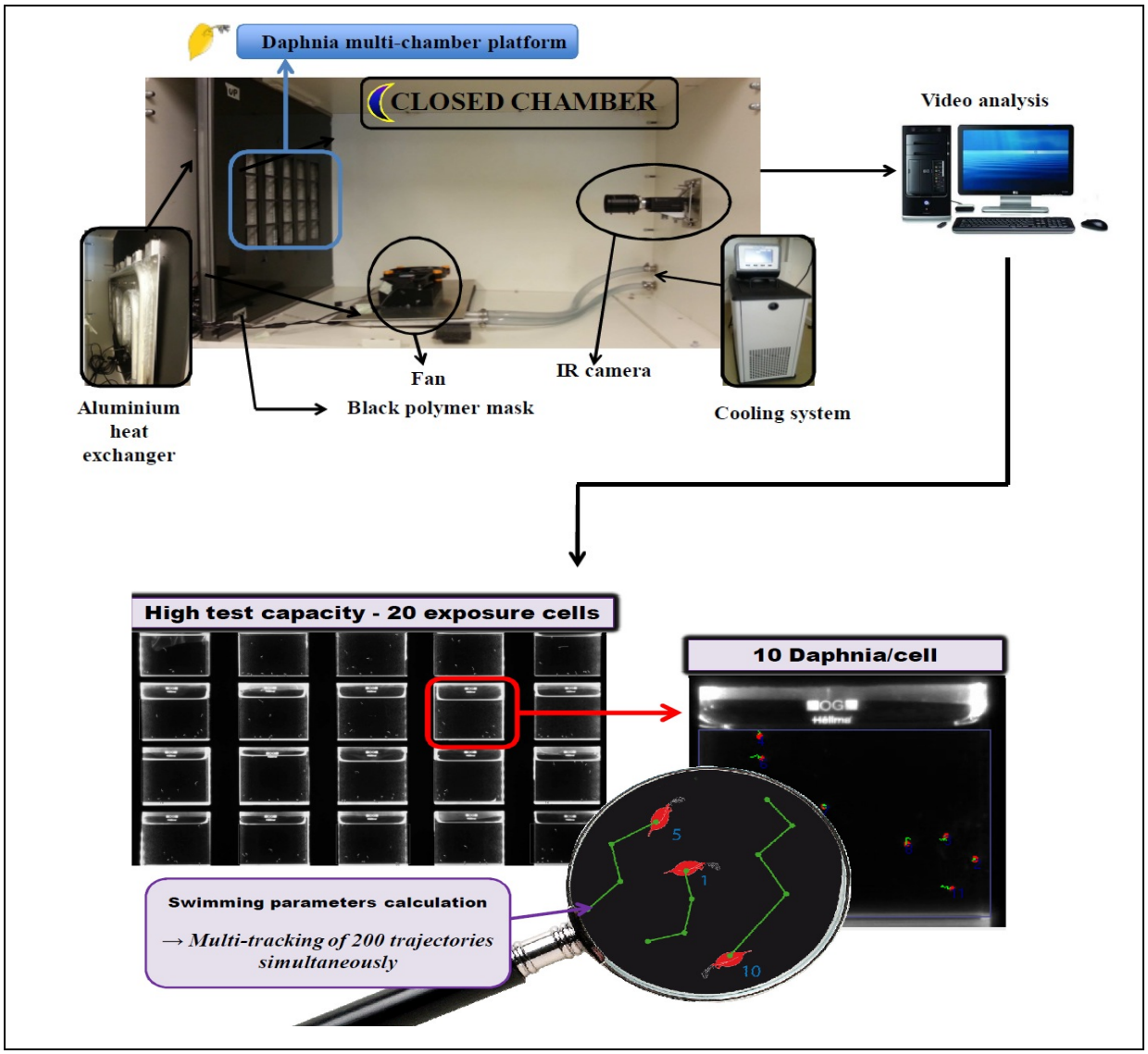

Figure 17: New behavioral multi-exposure cell test system named "Multi-DaphTrack". The exposure cell platform illuminated by Infra-Red light is placed in a closed chamber.

Daphnia magna culture and breeding and test organism preparation

Daphnia magna clones from parthenogenetic reproduction were reared in 1.5 L vessels containing artificial M4 culture medium at 20 ± 2°C with a photoperiod of 16 hours of light/8 hours of darkness in accordance with OECD test guideline 202 (2004). The M4 synthetic medium (OECD 2004) was prepared from an M4 concentrated solution (Life® Technology) that was diluted 10 times with ultra-pure water. M4 synthetic medium was then stabilized at pH 8. *Daphnia magna* females were fed daily with a batch-cultured unicellular algae suspension of *Chlorella vulgaris* ($3x10^5$ cells/mL ~ 0.1 mg C/daphnia/day quantified by a particle counter (Coulter Z1, Beckman & Coulter®)). To maximize neonate production, the vessels and M4 medium were replaced each week, 50% of the M4 medium was replaced twice a week, and filtration was performed each working day. To ensure

consistency with the acute immobilization test and reduce variability, cultures from 2 to 4 weeks were filtered the evening before and again on the day of the experiment to isolate neonates aged from 8 to 24 hours within the same size range. Selected neonates were then acclimatized in ISO standard artificial reconstituted freshwater ISO (OECD 2004) at 20°C without feeding.

Chemicals

Esfenvalerate (CAS: 66230-04-4) was purchased as a solid 100 mg powder (99% purity) from VWR®, France. Since esfenvalerate has very low solubility in water, the 250 mg/L stock solution was prepared by dissolving 25 mg of esfenvalerate powder in 100 mL of pure methanol. As recommended by the OECD (2000), the final concentration of methanol did not exceed 0.01% in the solvent control and the different assays. All tested solutions were prepared with the artificial reconstituted freshwater recommended by ISO 6341 standard (2012).

Esfenvalerate exposure

To define the concentration range for behavioral tests, an acute *Daphnia magna* toxicity test was first performed according to the OECD 202 test guideline (OECD 2004) and results are available in the supplementary data file (a). Based on these results, 5 nominal concentrations (0.14, 0.35, 0.88, 2.2 and 5.5 µg/L) were selected for the test in the multi-cell exposure system, and exposure conditions were designed to cover effect concentrations below EC_5 (48H) up to EC_{100} (48H) in the acute test. We used 3 replicates for each exposure treatment and 5 replicates for the controls (2 replicates of ISO water and 3 replicates of ISO water with 0.01% of methanol). Each exposure cell was filled with 20 mL of the test solution, sealed with PARAFILM "M" ® and kept at 20 ± 0.5°C (one additional exposure cell with ISO water was used to check the temperature in the exposure cell over time). 10 neonates were carefully and randomly placed in each test exposure cell. In addition to 48-hour video tracking, immobilization of daphnia was determined at the end of the 48-hour test. In order to compare the performance and sensitivity of the new test system, 4 concentrations (0.14, 0.35, 0.88 and 2.2 µg/L) were also tested in triplicate in the Daphnia toximeter (Bbe® Moldaenke, Kiel, Germany), a commercially available online biomonitoring system. 10 neonates were carefully placed in each 25 mL exposure cell. The specific conditions in the Bbe® Daphnia toximeter are described in the supplementary data file (b).

Statistical analysis

For the acute toxicity test, concentration-response curves of immobilization were modeled using the Hill model in the Regtox® macro for Microsoft Excel. Effect concentrations (EC_x) and their confidence intervals were estimated using the non-parametric "Bootstrap" method. As the behavioral data provided by the "Multi-DaphTrack" system are more complex and time-dependent, all further

statistical analyses were performed with customized scripts in the statistical software R (R 3.0.1). The signal was rather noisy; therefore to reduce the signal's noise and allow comparison between different concentrations, the average speeds per condition and per hour together with the variability were calculated. After verifying the compliance of the variance homogeneity and the normal distribution of data, a standard ANOVA model was performed with a simple student test by comparing each concentration to the control test ($p=0.01$) for each hour independently. Due to the test capacity constraint of the DaphToxI® system, only one control was performed in each experiment. For comparison of results, 12 controls were combined and considered as control reference. A linear mixed effect model was therefore applied to account for the day-to-day variability in the *Daphnia magna*'s average speed in the controls; the variability in speed was considered a random effect. This model was used to independently estimate the (fixed) effect of exposure with respect to the control condition for each hour.

RESULTS

Performance of the "Multi-DaphTrack" system

Optimization of the detection rate

Prior to optimization, detection rates under control conditions were below 50%, which was unacceptable. To improve detection of the small daphnia neonates (size comprised between 0.62 and 0.72 mm^2), illumination was firstly optimized: infrared LEDs were placed in each vertical row to increase the light intensity on each side of the measuring cells, ensure the homogeneity of the light and thus achieve optimal detection. Then, to avoid artefacts, light reflection was reduced with black adhesive strips, a background refresh option was performed frequently (every 60s) as a noise filtering operation and threshold detection parameters were also set up for each exposure cell independently. Lastly, the detection rate was around 93%, a result that proved to be consistent between measuring exposure cells and stable over time.

Control of experimental conditions in the "Multi-DaphTrack" system

Due to the distinct geometry of the exposure cells and the introduction of heat emitted by the light sources placed in close contact with them, substantial efforts were needed to stabilize the temperature and limit evaporation. Indeed, for illumination, 300 infrared 0.1W LEDs were placed close to the exposure cells, requiring the dissipation of 30W of thermal power to avoid a rise in temperature. The temperature in cells after 48 hours of exposure was equal to 26 ± 5.5 °C. A cooling system circulating water at 14°C with an aluminum heat exchanger and two ventilators was added to the exposure cell close to the LED strips. A stable and fixed temperature of 20 ± 0.5°C was finally obtained. Measurements of weight loss showed that water evaporation was negligible (< 1%) during

the 48-hour exposure period. The oxygen level in each cell with control medium was equal to 91.5 ± 1.5 % of the saturated oxygen concentration at the end of the exposure period, which is above the minimum of 60% as recommended by OECD (OECD 2004) and indicates no depletion of oxygen compared to the start of the experiment (saturated oxygen concentration). Besides, reference tests with the $K_2Cr_2O_7$ were regularly performed on Daphnids and results indicate that the sensitivity of the strain remains unchanged.

"Normal" behavior under control conditions

To determine the "normal" behavior of *Daphnia magna* under static conditions, 65 controls with non-exposed individuals were gathered from 13 different experiments (5 replicates per experiment). Throughout exposure, daphnids swam homogeneously with a path angle changing from 0 to ± 45°. The average ± standard error of the two parameters – mean velocity and number of active organisms are presented in Figure 18 for *Daphnia magna* exposed to control conditions over 48 hours.

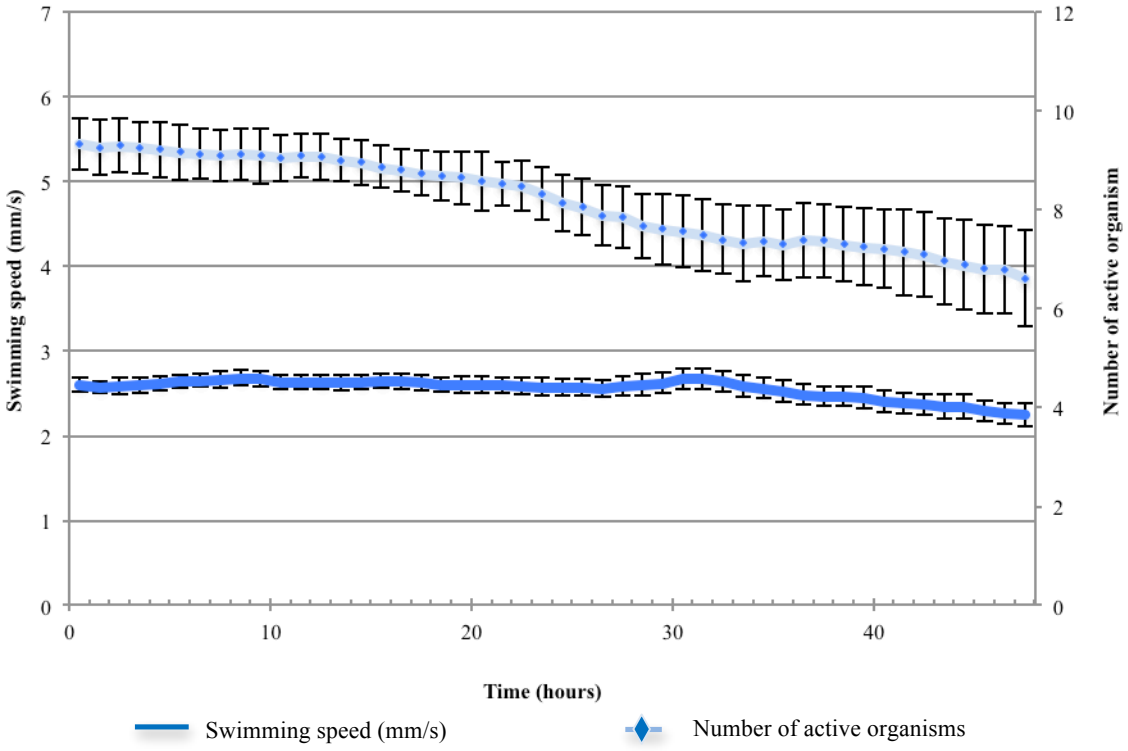

Figure 18: "Normal" behavior of *Daphnia magna* neonates under control conditions over 48 hours in the "Multi-DaphTrack" system at 20°C (mean values of n=65 replicates).

The mean swimming speed over the entire 48 hours of video tracking was equal to 2.55 ± 0.11 mm/s. Nevertheless, a slight decrease was observed at the end of the experiment (down to 2.24 mm/s). At the beginning, the number of active organisms detected by the system was around 9.3, which is consistent with the 10 daphnids introduced into each exposure cell. This slight under-detection was likely due to the loss of daphnids swimming close to the surface or to the walls of exposure cells or to crossing trajectories that are not recognized by the software's detection algorithm. During exposure, the number

of active organisms detected decreased constantly dropping to a level of 6.6 at 48 hours. No lethality was observed for daphnia under control conditions at the end of the 48 hours of exposure. This swimming activity is therefore not linked to mortality, but appears to reflect daphnia behavior in response to these exposure conditions. The number of active organisms was less uniform and showed higher variability between replicates compared to the swimming speed parameter, and the standard error increased at the end of exposure. It is also noted that the swimming speed remains stable while the number of active organisms tends to decrease between the 20th and the 35th hours; thereafter the swimming speed also decreased. Resting time, defined as any period of inactivity, was also measured, but no significant pattern was demonstrated under control conditions. No lethality was observed at 48 hours after gentle agitation, meaning that neonates did not die during the experiment but were merely in a state of rest. Result on the influence of daphnia density on swimming speed showed that except for the swimming speed of a single daphnia (averaged at 1.71 ± 0.13 mm/s over 48 hours), there was no significant difference between the mean swimming speed of a given group of 3 up to 15 individuals (averaged at 2.31 ± 0.06 mm/s). Therefore density does not appear to modify daphnia swimming speed (except for isolated individuals).

Esfenvalerate exposure results

Test in the "Multi-DaphTrack" system

Swimming speed, number of active organisms and change in path angle parameters were measured for each exposure condition. Nevertheless, since the number of active organisms and changes in path angle parameters did not add much useful information concerning toxicity, the average swimming speed parameter has been selected to assess behavioral effects. Time courses for the swimming speed for control and esfenvalerate-exposed *Daphnia magna* and a histogram of increasing and decreasing speed effects relative to controls over time are shown in Figure 19. Overall, esfenvalerate induced a significant increase in swimming speed at all tested concentrations after the first hour of exposure (from 23 to 107% above the control level). A slight, but significant increase in swimming speed (+ 23% relative to the controls, $p< 0.01$) was observed for the lowest test concentration of 0.14 μg/L (below EC_5 (48H)) after the first hour of exposure and lasted 16 hours. The swimming speed then returned to the control level. The most pronounced increase in swimming speed was observed at 0.35 μg/L (near EC_{10} (48H)) with the maximum speed occurring at 4 hours (average speed increase was 123% ($p< 0.01$) above control level). This exposure concentration caused excitation of the daphnia for 21 hours before the swimming speed returned to the control level (longest excitation effect duration observed in the experiment). A similar sharp increase was observed for concentrations of 0.88 μg/L (near EC_{50} (48H)) and 2.2 μg/L (above EC_{70} (48H)) with a maximum increase of 117% reached at 2 hours. The excitation effects lasted 15 hours for the 0.88 μg/L concentration and 6 hours for the 2.2 μg/L concentration until the swimming speed dropped

significantly below control level from 28 hours and 15 hours respectively. A smaller, but significant increase in swimming speed compared to the controls occurred for the 5.5 µg/L concentration (maximum of 63% attained at 1 hour ($p< 0.01$)), but did not last more than 3 hours. Then a significant decrease compared to the control group (around 45%, $p< 0.01$) was observed at 8 hours of the experiment. At the end of the 48 hours of exposure, 100% immobilization of daphnids was observed. The 48 hours EC_{50} for *Daphnia magna* immobilization exposed to esfenvalerate was calculated to be 1.04 ± 0.01 µg/L in the multi-cell exposure system, which is consistent with the results of the standard acute toxicity test (EC_{50} (48H) = 0.89 ± 0.12 µg/L).

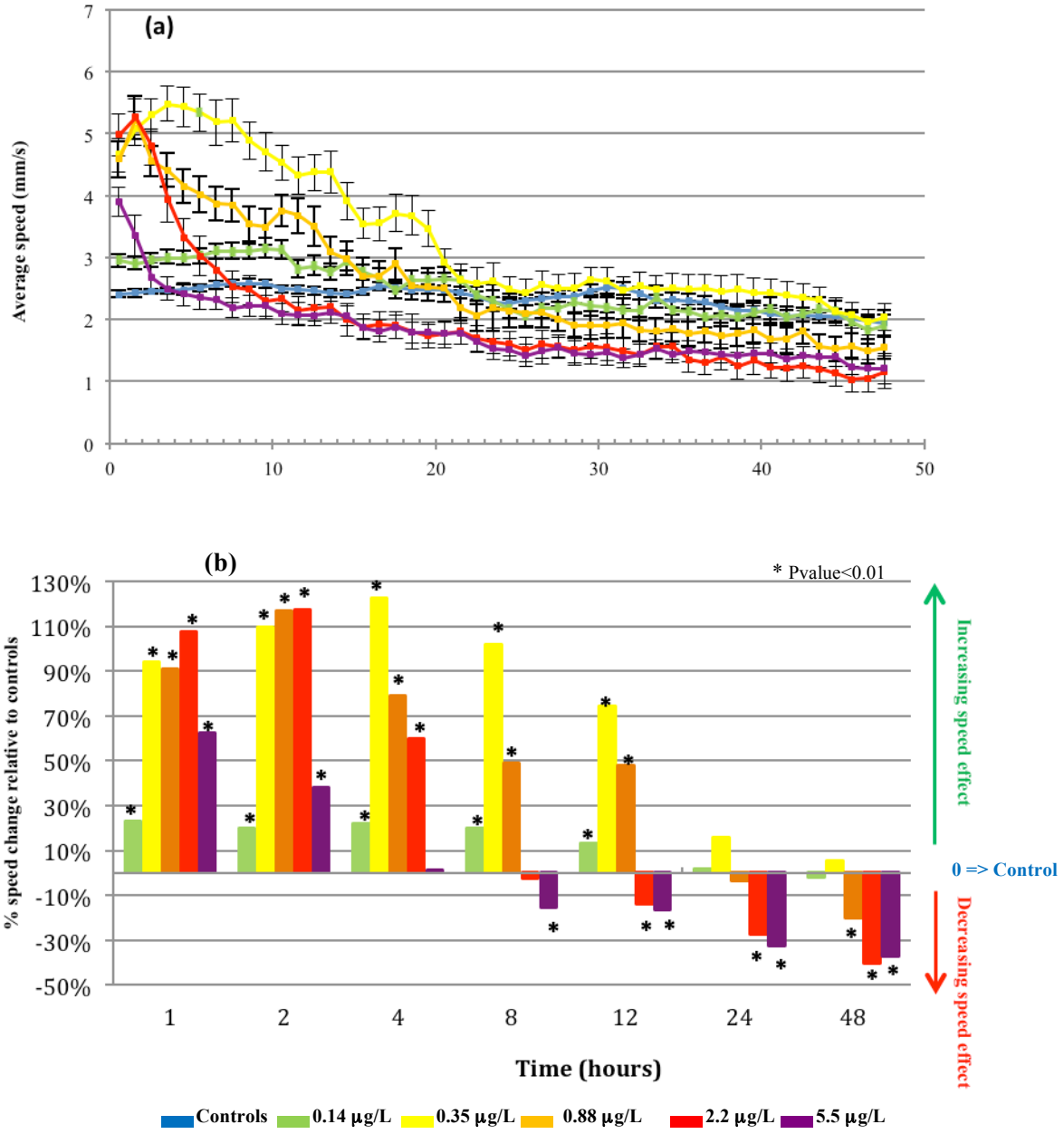

Figure 19: (a) Average swimming speed (per hour) (b) Histogram of increasing and decreasing speed effects relative to the controls over time for *Daphnia magna* exposed to different concentrations of esfenvalerate in the "Multi-DaphTrack" system.

The time course of the average swimming speed of the control and esfenvalerate-exposed *Daphnia magna* over time in the Bbe® Daphnia toximeter is shown in Figure 20 (a).

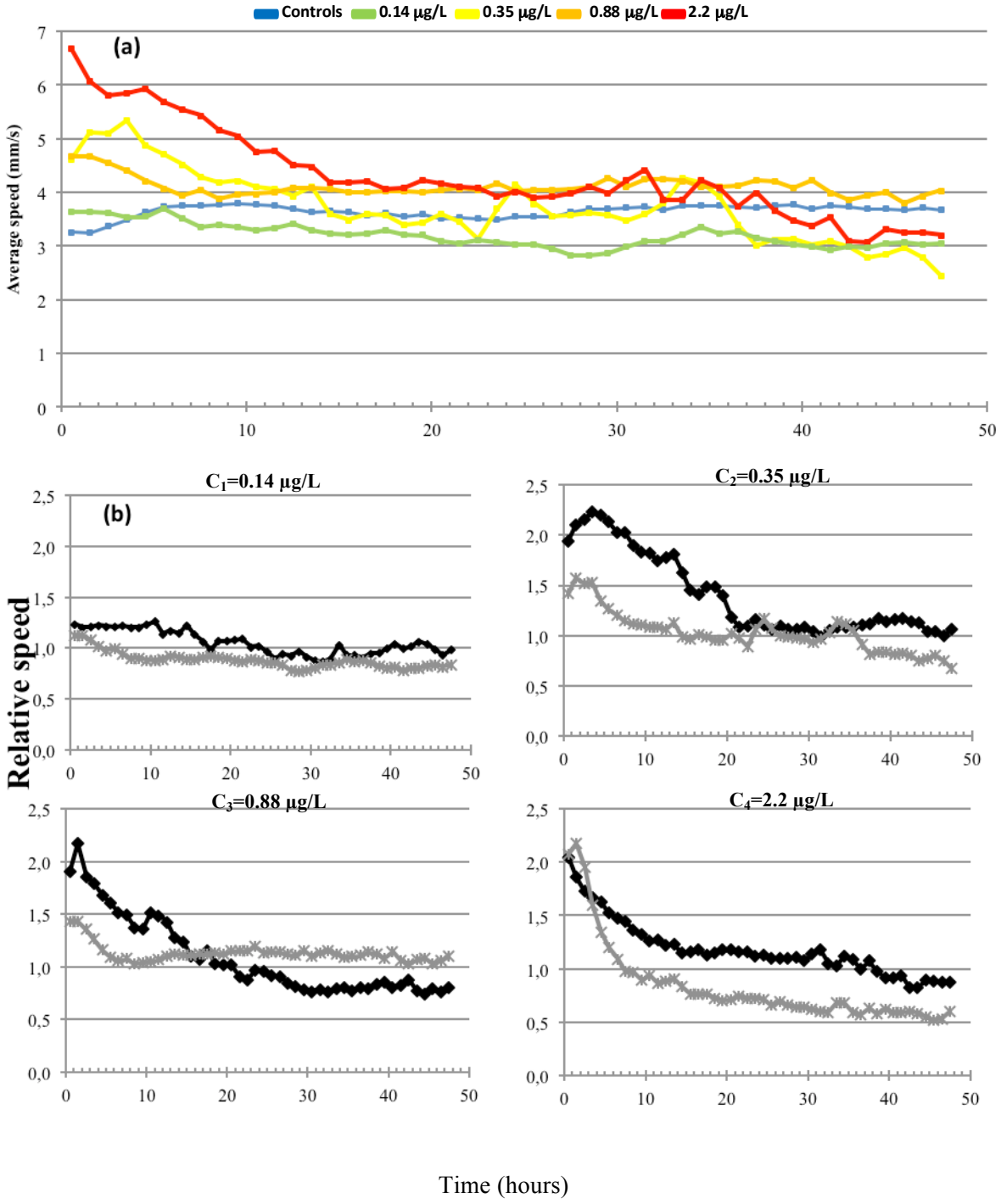

Figure 20: (a) Average swimming speed (per hour) of *Daphnia magna* exposed to several concentrations of esfenvalerate for 48 hours on a Bbe® Daphnia toximeter, (b) Comparison between relative swimming speeds (per hour) of *Daphnia magna* exposed to several concentrations of esfenvalerate during 48 hours in the Bbe® Daphnia toximeter and in the multi-cell exposure test system, legend: Black: relative speed in the "Multi-DaphTrack" system, Grey: relative speed in the Bbe® Daphnia toximeter.

In the Bbe® Daphnia toximeter, an average swimming speed of 3.65 ± 0.13 mm/s was observed for control conditions over a period of 48 hours: daphnids swam 1.5 times faster in the Bbe® Daphnia toximeter than in the "Multi-DaphTrack" system (2.35 ± 0.18 mm/s). While the swimming speed slightly decreases over time in the "Multi-DaphTrack" system, the average swimming speed remained stable in the Bbe® Daphnia toximeter. No significant effect on swimming speed was observed for the lowest tested concentration of esfenvalerate at 0.14 µg/L (below EC_5 (48H)). A significant increase in swimming speed ($p < 0.01$) was observed from the first hour lasting 14 hours for daphnia exposed to 0.35 µg/L (below EC_{10} (48H)). A slight, but significant increase in swimming speed ($p < 0.01$) was measured for 0.88 µg/L (near EC_{50} (48H)) and lasted 3 hours. The highest significant increase in swimming speed was observed for the highest concentration of 2.2 µg/L (above EC_{70} (48H)) and lasted 10 hours.

Comparison of the "Multi-DaphTrack" and the Bbe® Daphnia toximeter

Relative speeds (i.e. ratio of the average swimming speed of daphnids exposed to esfenvalerate divided by the average swimming speed of the controls) were calculated for each concentration for the comparison between the "Multi-DaphTrack" and the Bbe® Daphnia toximeter and are presented in Figure 20 (b). On the whole, relative speeds follow the same trends (i.e. rapid peak increase) except for the first tested concentration of 0.14 µg/L. The most pronounced increase is observed for the 0.35 µg/L tested concentration (below EC_{10} (48H)).

DISCUSSION

Validation of the new "Multi-DaphTrack" system

Several biotic and abiotic factors such as age, temperature and light may have a significant impact on daphnia behavior. Great effort was therefore made to control the experimental conditions inside the new "Multi-DaphTrack" system. For instance, infrared light, which is not visible to daphnia, was chosen to prevent any light-induced behavior disturbances. Moreover, temperature which is one of the most important abiotic factors able to influence several physiological and biological processes in all organisms (Gordon 2003) was tightly controlled. For instance, when temperature rises, animals swim up more frequently (Gerritsen 1982). Low temperatures (e.g. 0 to 5°C) on the other hand can greatly decrease swimming ability of *Daphnia magna* (Chen et al. 2012). Temperature is also a potential stressor in itself, which may modify toxic effects in daphnids and other species (Holmstrup et al. 2010, Messiaen et al. 2010, Muyssen et al. 2010). Furthermore, although detection errors cannot be avoided entirely (e.g. cross-over swimming, detection loss), our optimization work of illumination and adjustments to the software parameters have resulted in an acceptable detection rate of 93% for the new "Multi-DaphTrack" system.

Results of "Normal" behavior showed that daphnids swam homogeneously over time (stable average speed and number of active organisms parameters) despite a decrease of both parameters at the end of experiment. This decrease in both parameters can be explained by the exposure conditions: static conditions do not stimulate daphnia movement and neonates were not fed throughout the whole experiment and likely became tired and in need of rest. By watching the video and the tracking during the experiment, we can confirm that neonates alternated swimming with periods of rest at the end of exposure. This decrease in daphnia activity is not due to a decrease in the detection rate. Furthermore, the software is incapable of detecting small movements such as turns and gyres along the main body axis for numerous active organisms. As a result, we cannot consider the number of active organisms parameter to be entirely similar to the standard immobilization parameter over time. Otherwise, organisms may interact with each other and the density of the organisms inside the measuring cell may affect behavioral parameters. However, our results showed that density does not modify daphnia swimming speed (expect for isolated individuals). Therefore, our results ensure that when mortality occurs during exposure to acute toxic concentrations of chemicals, the death of some individuals does not influence the swimming speed of the remaining living individuals.

Effects of esfenvalerate on Daphnia magna behavior

Behavior disturbances can be considered as a sequence of neuro-sensorial, muscular disruption, sensorial, energy disturbances and muscular contraction events (Amiard-Triquet and Amiard 2013, Lagadic et al. 1994, Untersteiner et al. 2003). Many pesticides are known to be extremely toxic to aquatic organisms and although esfenvalerate is generally applied at low doses, non-target aquatic organisms are highly sensitive to even low levels of this insecticide (Tang and Siegfried 1995). For instance, significant alteration of the behavior was previously reported on the fathead minnow (*Pimephales promelas*) (Floyd et al. 2008) and on the arthropods Black margined Aphid, Black Pecan Aphid, and Yellow Pecan Aphid (Hure and D. Dutcher 1994). Our results showed that the toxic effects of esfenvalerate on behavior increase as the exposure concentration increases. A rapid increase in the swimming speed of *Daphnia magna* occurred from the first hour of esfenvalerate exposure. This effect is consistent with the mode of action of esfenvalerate, which modulates sodium channel activity. It induces the reversible blocking of sodium channels in the neurons, leading to impaired action potential along the axon and hyper-excitation followed by muscle paralysis and death by respiratory arrest (Hodgson 2004). Another hypothesis is that daphnids try to escape from the polluted area by increasing their swimming speed (hyperactivity) as a protective avoidance response (Amiard-Triquet and Amiard 2013). In the "Multi-DaphTrack" system, the speed increase is followed by a decrease back to the control level for sub-lethal concentrations up to 0.35 µg/L (below EC_{10} (48H)). For concentrations of 0.88 µg/L (near EC_{50} (48H)) and above, swimming speed declined over time to levels two times lower than the control levels. Recovery at low concentrations is probably due to esfenvalerate reversible mechanism of action and the fact that esfenvalerate exerts low acute effects

at those concentrations. Detoxification mechanisms may also be involved, leading to rapid elimination of the toxin and a return to normal behavior. The speed decrease for acute concentrations (>=0.88µg/L) suggests a loss of energy for muscle activity or locomotion and may be explained by tiredness and/or the first signs of harmful effects (Jeon et al. 2008). These results corroborate previous studies where significant alterations in *Daphnia magna* behavior were detected for higher concentrations (Lewandowska 2004) and for a similar cypermethrin concentration range, a chemical that shares the same mode of action as esfenvalerate (Werth 2006).

On the whole, the behavior effects induced by esfenvalerate exposure observed in the "Multi-DaphTrack" system follow the same trends as in the Bbe® Daphnia toximeter with a similar increase in swimming speed from the first hour of exposure. However, although the average swimming speed in the Bbe® Daphnia toximeter was higher under control conditions, esfenvalerate effects were less pronounced in the Bbe® Daphnia toximeter compared to the "Multi-DaphTrack" system. Moreover, the duration of the effect was shorter in the Bbe® Daphnia toximeter.

Sensitivity of the "Multi-DaphTrack" system

Organisms reacted shortly after exposure to esfenvalerate, with a significant increase in swimming speed from the first hour of exposure to the lowest behavioral effect concentration of 0.14 µg/L. The results show that this increase in swimming speed is a very sensitive and early behavioral response to multi-stress including pollutant exposure. Behavioral disturbances have already been reported in daphnids exposed to sub-lethal concentrations of xenobiotics such as cadmium and copper (Baillieul and Blust 1999, Untersteiner et al. 2003, Wolf et al. 1998). Furthermore, this system would be sensitive enough to detect effects induced by environmental concentrations of esfenvalerate such as 0.66 µg/L measured in Danish streams (Cold and Forbes 2004) or around 0.15 µg/L found in American river water (Brady et al. 2006). With this in mind, tests on substances with different modes of action can be performed to see if the system is equally sensitive to a wide range of chemicals with different modes of action. This system offers the potential of monitoring effects over a period of 48 hours that is of significant relevance for substances with delayed effects.

To conclude, this "Multi-DaphTrack" system shows a higher sensitivity compared to a conventional immobilization assay with significant responses detected below EC_5 (48H). The "Multi-DaphTrack" system is also more sensitive than the Bbe® Daphnia toximeter. This is mainly due to the fact that the number of replicates in the "Multi-DaphTrack" system provides more robust results and decrease variability. *Daphnia magna* swimming speed can likely be considered as a rapid and sensitive indicator of toxic stress. One interesting aspect of the swimming speed parameter is that it is not only sensitive compared to the traditional acute toxicity test, but it also responds quickly after the start of exposure, while lethal effects can be delayed. Overall, the "Multi-DaphTrack" system gives the possibility to successfully record simultaneously the behavior of 200 daphnids in different chambers at

the same time. This "Multi-DaphTrack" system can be used to detect very sensitive and significant behavioral responses of toxic stress and hence reinforce primary results provided by early but less sensitive BEWS systems.

ACKNOWLEDGMENT

This work was supported by EDF in collaboration with Bordeaux1 University and INERIS, France. We thank S. Maestri, the technical personal and E. Harscoët for the help in the experiment, U. Azmi and D. Chadaigne to help developing script on R for data treatment and statistical analysis.

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CHAPTER III. EXPLORATION OF DAPHNIA BEHAVIORAL PROFILES INDUCED BY A BROAD RANGE OF TOXICANTS WITH DIFFERENT MODES OF ACTION

1. PARAMETERS MEASURED IN THE "MULTI-DAPHTRACK" SYSTEM

The number of active organism parameter was also measured for the twelve substances in the "Multi-DaphTrack" system. As discussed in the **chapter II**, the study was mainly focused on the average speed parameter for toxicity measurement and the number of active organisms was not exploited in articles. Representative results of the effects on the number of active organism parameter induced by chemical exposure are presented in the Figure 21.

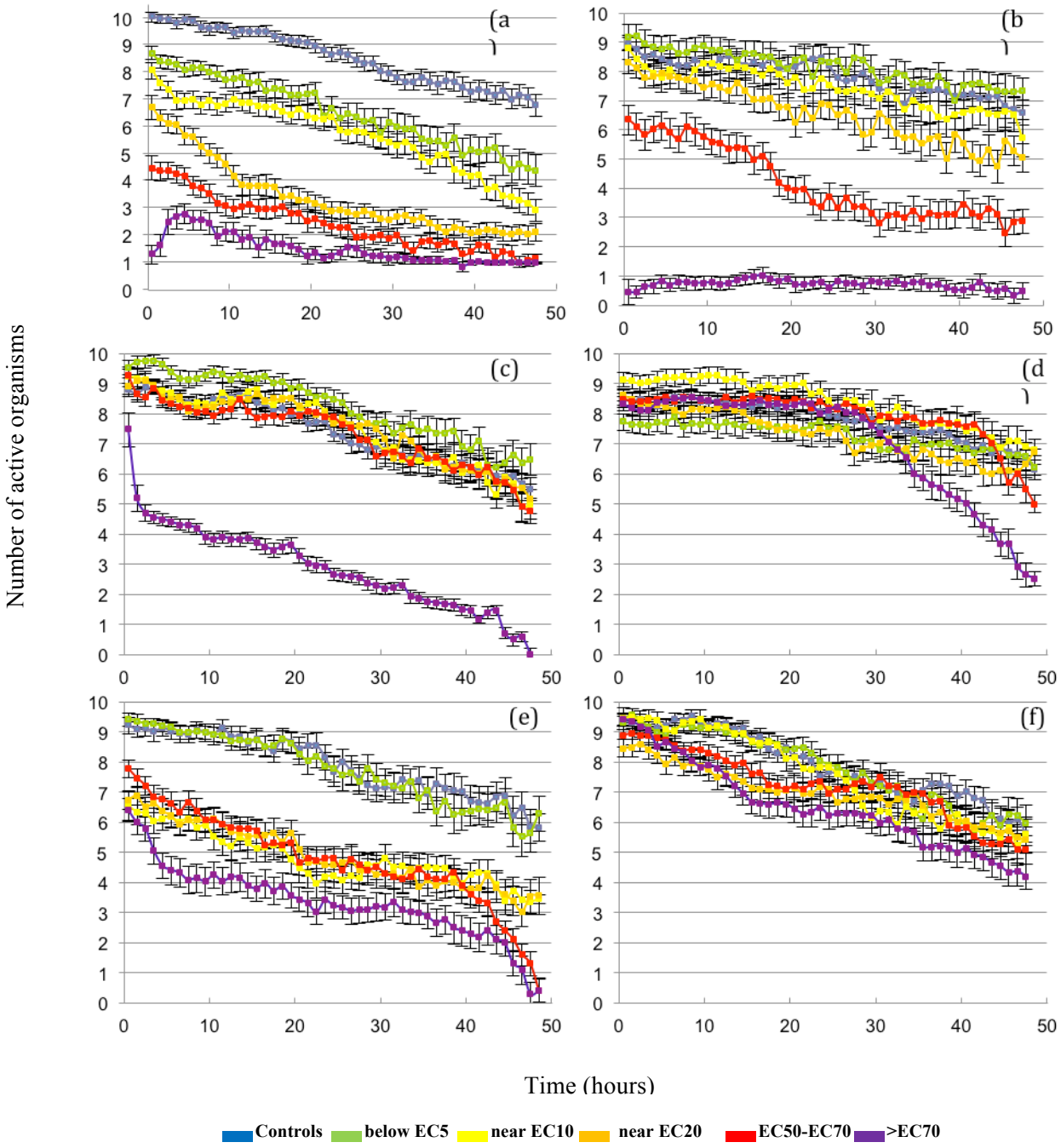

Figure 21: Average number of active organisms (per hour) of *Daphnia magn*a exposed to several concentration of (a) isopropanol, (b) ethanol, (c) sulfate copper pentahydrate, (d) trichlorfon, (e) esfenvalerate and (f) fipronil in the "Multi-DaphTrack" during 48 hours.

Overall, these results corroborate that the number of active organisms do not add such interesting information about toxicity. As the average speed, the number of active organisms gradually decreases over time for the narcotic isopropanol exposure. All tested concentrations of isopropanol (from 1.3 to 13.9 g/L, i.e., $<EC_5$ to $>EC_{70}$ (48 h)) induced significant decrease effects on the number of active organisms parameter from the first hour of exposure until the en of the 48 h of exposure. Ethanol induced similar but slighter decrease effects on the number of active organisms. Significant decrease effects were only observed from the concentration of 4.8 g/L (between EC_{10}-EC_{20}), for which the decrease effect gradually increase from 13% at 11 h to 31% at 48 h. The concentration of 12 g/L induce a decrease of 41% from the first hour of exposure and reached 139% at 48 h of exposure. For the highest concentration of 30 g/L, the number of active was nearly equal to 0, which corroborate with the results of the swimming speed (near to 0 mm/s). At this concentration, 100% of immobilization was observed at the end of 48 h and obviously indicate mortality that probably occurred from the first hour of exposure. For copper sulfate exposure, significant decrease effects on the number of active organism parameter were only observed for the highest concentration of 363.5 µg/L ($>EC_{70}$) with 18% of decrease effect at the first hour of exposure to 264% of decrease effect at the end of exposure. Trichlorfon exposure only provoked significant effect on the number of active organisms for the highest tested of 0.336 µg/L (EC_{70} 48 h): a decrease effect of 23% was observed from 34 h and reached 143% at the end of exposure. This effect of decrease was very delayed, as for the average speed. For esfenvalerate exposure, the two concentration of 0.352 and 0.88 µg/L (EC_{10} and EC_{50} respectively) induced a similar intense and significant decrease of approximately 39 % at the first hour of exposure and then the decrease effect gradually increases until to reach 70% at the end of exposure. The number of active organisms also significantly decreased for the two highest concentrations ($>EC_{70}$) and was comprised between 0 and 1 at the end of exposure, which correlate with the average speed of 1 mm/s at the end of exposure. Finally, slight but significant effects on the number of active organism were observed for the tested concentrations of 7 µg/L (EC_{20}), 15.4 µg/L (between the EC_{20}-EC_{50}) between 1 to 19 hours and then the number of active organism went back to control level. The highest tested concentration of 33.9 µg/L (EC_{50}) induced significant decrease effects during all the exposure time. These results corroborate with the recovery observed after an induced increase average speed in the first hours of exposure of fipronil.

ARTICLE 2 - EXPLORATION OF DAPHNIA BEHAVIORAL EFFECT PROFILES INDUCED BY A BROAD RANGE OF TOXICANTS WITH DIFFERENT MODES OF ACTION

Effects of different toxicants on daphnia behavior

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ABSTRACT: Behavior is increasingly reported as a sensitive and early indicator of toxicant stress in aquatic organisms. However, the systematic understanding of behavioral effects and comparisons between effect profiles is hampered because the available studies are limited to few chemicals and differ in the exposure conditions and effect parameters examined. The aims of this study were (i) to explore behavioral responses of *Daphnia magna* exposed to different toxicants, (ii) to compare behavioral effect profiles with regard to chemical modes of action and (iii) to determine the sensitivity and response time of behavioral parameters in a new multi-cell exposure system named "Multi-DaphTrack" compared to currently utilized tests. Twelve compounds covering different modes of toxic action were selected to sample a wide range of potential effect profiles. Acute standard immobilization tests and 48 h of behavioral tracking were performed in the customized "Multi-DaphTrack" system and a single-cell commercialized biological early warning system (BEWS). Contrasting behavioral profiles were observed for average speed (i.e., intensity, time of effect onset, effect duration), but no distinct behavioral profiles could be drawn from the chemical mode of action. Most compounds tested in the "multi-DaphTrack" system induced an early and significant average speed increase at concentrations near or below the EC10 (48 h) of the acute immobilization test, demonstrating that the "Multi-DaphTrack" system is fast and sensitive. To conclude, behavior endpoints could be used as an alternative or complement to the current acute standard test or chemical analysis for the predictive evaluation of ecotoxic effects of effluents or water bodies.

KEYWORDS: *Daphnia magna*, behavioral tracking systems, behavior, effect-based tool, chemical modes of action

INTRODUCTION

To assess the hazard and the risk of chemicals released in aquatic environments, toxicity measurements using standardized tests on representative organisms from different trophic levels (fish, daphnia and algae) are usually performed. The acute immobilization test on *Daphnia magna* ISO 6341 (2012), OECD (2004) is one of the most frequently used standardized tests and typically serves as reference for chemical risk assessment. *Daphnia magna* is highly sensitive to a wide range of chemicals and is representative of freshwater organisms (Mark and Solbé 1998). However, in the standard protocol, immobilization is measured as an effect parameter at two pre-defined exposure times of 24 h (usually for effluent assessment) and 48 h (for other substances); these measurements are not sensitive enough to account for sub-lethal effects that can be induced by low concentrations that typically occur in surface waters. Indeed, concentrations inducing mortality are fortunately infrequent in aquatic environments; a recent large-scale study encompassing 4000 European monitoring sites revealed that concentrations above 1/10 and 1/1000 of the EC_{50} (48 h) level for *Daphnia magna* were observed at 6 and 38% of the sites, respectively (Malaj et al. 2014). To protect and/or restore aquatic ecosystems, the rapid assessment of the toxic potency of environmental pollutants is required (Hellou 2011). Behavioral tests may provide earlier responses compared to both acute/chronic standard tests since toxic stress can induce rapid behavioral changes in exposed organisms at concentrations below the acutely toxic levels (Amiard-Triquet and Amiard 2013). Behavior is a sensitive indicator of acute/sub-lethal toxicity (Coelho et al. 2011) and may be suited to detecting stress induced by realistic environmental concentrations of pollutants. Hence, the use of behavioral response data could improve the evaluation process of environmental risk assessments (Robinson 2009).

Swimming behavior is primarily measured in the laboratory to characterize adverse behavioral effects from a single chemical exposure. Several video tracking systems under static conditions have been developed to analyze alterations in daphnia swimming parameters after exposure to metals (Jeon et al. 2008, Untersteiner et al. 2003), pesticides (Zein et al. 2013), nanoparticles (Artells et al. 2013), water field samples and cyanobacteria (Ferrao-Filho et al. 2014). Nevertheless, the daphnia behavior analysis systems currently available on the market offer a limited test capacity that does not allow for a systematic analysis of the robustness of behavioral endpoints. To investigate whether daphnia behavior endpoints can be used as tools for ecotoxicological assessments of water quality, the behavior of daphnia must be understood by testing different chemicals under controlled conditions at several concentrations and replicates to allow for appropriate statistical analysis. Therefore, a new daphnia behavioral analysis system with a high test capacity and named “Multi-DaphTrack” (Chevalier et al. 2014) has been previously developed using recent advances in video tracking technology; to simultaneously monitor the trajectories of a large number of daphnia groups with a high number of replicates. This system has already provided sensitive and early behavioral responses with the insecticide esfenvalerate (Chevalier et al. 2014).

Several behavioral measurements are also currently performed *in situ* with biological early warning systems (BEWSs) to provide real-time information on water toxicity. Several commercialized BEWSs use daphnia (e.g., the daphnia toximeter DaphTox® (Bbe® Moldaenke, Kiel, Germany), and the Multi Freshwater Biomonitor MFB® (LimCo®, Konstanz, Germany)) and have been used for two decades as an alternative or complement to the conventional water quality monitoring approach (i.e., chemical analysis and ecotoxicity assays) to detect toxic pollution peaks along rivers or downstream of effluents (Gerhardt et al. 2006). In several cases, such systems were able to detect pollutants that were not detected by traditional

chemical analysis systems (De Hoogh et al. 2006). For instance, the DaphTox® (Bbe® Moldaenke, Kiel, Germany) system was successfully used as a BEWS for detecting chemical pollution in surface water or intakes for drinking water production in northern Europe (Werth 2006) or in rivers affected by industrial activities in China and North Korea (Van Den Broeke 2013). Alarm threshold concentrations in daphnia BEWSs inducing detectable behavioral changes have been established for numerous isolated chemicals and are currently used as a reference by operators of the DaphTox® (De Hoogh et al. 2006, Lechelt 2006, Wiklund et al. 2012) and MFB® (Ren et al. 2009 (a), Ren et al. 2007, Ren et al. 2009 (b)) systems. However, despite substantial efforts undertaken by various operators, no systematic analysis of behavioral changes induced by a large range of toxics has been performed.

Behavior is increasingly reported as a sensitive and early indicator of toxicant stress in aquatic organisms. However, the systematic understanding of behavioral effects and comparisons between effect profiles is hampered because available studies are limited to select chemicals, typically analyzed for a specific assessment purpose, that differ in exposure conditions and effect parameters. Additionally, standardization and field validation of these behavioral responses are still lacking, and the characterization of the chemical concentration that can induce behavioral effects is not straightforward. Consequently, it is currently unclear how to quantify the sensitivity of behavioral endpoints, which limit their use as regulatory criterion in risk assessment or water quality monitoring. To meet the objectives of recent environmental regulations such as the Water Framework Directive (WFD) and the Registration, Evaluation and Authorization of Chemicals (REACh), efficient ecotoxicological testing is needed in risk assessment, and improved testing strategies are recommended (Breitholtz et al. 2006). With the numerous chemicals potentially present in the environment, the screening of behavioral effects of all aquatic pollutants is not realistic. Selecting test chemicals according to their mode of action may potentially facilitate the extrapolation of observed behavioral effect profiles and the time of effect onset to compounds with a similar mode of action. Theoretically, compounds sharing a mode of action should behave in a similar manner and should show similar toxic effects and thus behavioral effects (Zein et al. 2013). Behavioral studies that are based on the investigation of various modes of action have been reported for fish (Drummond and Russom 1990), but similar studies have, to our knowledge, not been performed for *Daphnia magna*. In

the present study, an exploratory investigation of the behavioral effects induced by pollutants was conducted by selecting test chemicals with a wide range of different modes of action. This study represents, to our knowledge; the first reported multiple-toxicant behavioral study of the most commonly used species for assessing the hazard of chemicals (i.e., *Daphnia*).

The aims of the study were (i) to investigate various behavioral responses of *Daphnia magna* exposed to chemical with different modes of action, (ii) to determine whether the behavioral response profiles measured could be distinguished based on the chemical mode of action and (iii) to determine how early and sensitive the behavioral endpoints are in regard to the standard immobilization endpoints. *Daphnia magna* neonates were exposed to a range of concentrations of twelve compounds with different modes of toxic action to sample a wide range of potential effect profiles. Immobilization acute toxicity tests were also performed on the twelve substances to evaluate the sensitivity of the "Multi-DaphTrack" system by comparing behavior effect results to the EC_{50} (48 h) of the classical acute toxicity test. Furthermore, to compare behavior trends from our "Multi-DaphTrack" system to a commercialized BEWS currently used in biomonitoring programs, one sublethal concentration for each chemical was tested in the DaphToxI® system (Bbe® Moldaenke, Kiel, Germany).

MATERIALS AND METHODS

Daphnia magna culture and test organism preparation

Daphnia magna clone A (from IRCHA, France) from parthenogenetic reproduction was reared in 1.5 L vessels containing artificial M4 culture medium at 20 ± 2 °C. The photoperiod was 16 h of light and 8 h of darkness, as recommended by OECD test guideline 202 (OECD 2004). The M4 medium was prepared from an M4 concentrated solution (Life® Technology), which was diluted 10-fold with pure Milli-Q water and then stabilized at pH 8. The *Daphnia magna* colony was fed daily with a batch-cultured unicellular algae suspension of *Chlorella vulgaris* harvested from a non-synchronous culture (3 x 105 cells/mL, ~ 0.1 mg C/daphnia/day quantified by a particle counter (Coulter Z1, Beckman & Coulter®)). To maximize daphnia neonate production, the vessels and M4 medium were renewed each week, 50% of the M4 medium was replaced twice per week, and filtration was performed each working day. To ensure consistency between the acute immobilization test and the behavioral assay and to minimize variability, cultures from 2 to 4 weeks were filtered the evening before and again on the day of the experiment to isolate neonates aged from 8 to 24 hours that were within the same size range. Selected neonates were then acclimatized in an ISO standard artificial reconstituted freshwater (OECD 2004) at 20°C without feeding for 2 h.

Chemical selection to cover a range of modes of action

Test compounds were selected to cover different modes of action: two narcotics (ethanol and isopropanol), two acetylcholine esterase inhibitors (organophosphate trichlorfon and the carbamate carbofuran), an agonist of the nicotinic acetylcholine receptor (neonicotinoid imidacloprid), an agonist and an antagonist of the GABA receptor (avermectin abamectin and the phenylpyrazole fipronil, respectively), two modulators of sodium channels (pyrethroids esfenvalerate and cypermethrin), a xanthine alkaloid (caffeine), and a psychotropic drug acting as a selective serotonin reuptake inhibitor (sertraline). Finally, copper sulfate pentahydrate was tested as a representative compound with multiple modes of action. In this present study, the modes of action assignments were evaluated and classified by gathering information mainly from the pharmaceutical/toxicological literature and supported with toxicodynamic knowledge. Chemical details are available in the supplemental data file.

Acute toxicity tests

Daphnia immobilization acute toxicity tests were primarily conducted on the twelve substances at 20 ± 2°C in darkness according to the OECD 202 test guideline (OECD 2004). Five tested concentrations were selected according to the EC_{50} (immobilization) values found in the literature, and the concentration range was designated to include 0 to 100% immobilization with a dilution factor below 2.2. The objective of these tests was to facilitate the selection of sub-lethal exposure concentrations for behavioral experiments. Furthermore, $EC_{50,baseline}$ values were calculated using a QSAR equation (1) by assuming a narcotic mode of action in *Daphnia magna* (Zhang et al. 2013). To compare the experimentally determined toxicity ($EC_{50,exp}$) with the "baseline toxicity" ($EC_{50,baseline}$) and to quantify how much more toxic a compound is compared to acting via a narcotic mode of action alone, the excess toxicity, Te, was calculated according to equation (2) (von der Ohe et al. 2005). A log Te between -1 and 1 indicates inert or less inert toxicity compounds, whereas a log > 1 reveals an excess toxicity (specific mode of action).

$$Log\left(\frac{1}{EC50,baseline}\right) = 0.824 \log Kow - 1.58 \quad (1)$$

$$Te = \frac{EC50,baseline}{EC50,exp} \quad (2)$$

Behavioral tests performed with the "Multi-DaphTrack" system

This system has been designed to share the same experimental condition as the standard immobilization test: static exposure, darkness and room temperature maintained at 20°C. In total, 20 exposure cells (20 mL) containing 10 daphnia each can be analyzed in parallel. Further details on the systems are available in Chevalier et al. (2014). The video was recorded and transferred to a computer in AVI format. The video was then analyzed using the Zebralab® software algorithms (Viewpoint®,

Life Technology) which allows to extract the XY coordinates of daphnia neonates by tracking each Daphnia's center of gravity at each frame (25 frame/s) and to reconstruct trajectories. Three behavioral parameters were calculated for each measuring cell (group of 10 daphnids): swimming speed, number of active organisms and path angle. Averaged parameters were finally calculated per hour over 48 h and saved in an Excel file. This exposure time was selected to directly compare the results with the endpoints of the standard tests. For each concentration, the time course of average speed and the time of effect onset were compared to control group responses to quantify behavior changes induced by chemicals. The average speed in *Daphnia magna* neonates following toxicant exposure had a higher sensitivity than the other behavioral endpoints in our "Multi-DaphTrack" system (2014). Hence, the following study will only focus on the average speed parameter. A toxicokinetic index was also calculated by simply multiplying the lowest observed effect threshold for the average speed by the time effect onset and expressed in mg h L-1,

Five concentrations were then selected for behavioral tests in the "Multi-DaphTrack" system for each tested compound to cover the range from no observable immobilization to a high level of immobilization. Prior to the experiment, the optimal number of replicates for controls and for each contaminant concentration with the 20 cells available was calculated following Dunnett's optimal distribution (the "square root rule"). Thus, 3 replicates for each of the 5 concentrations and 5 replicates for the controls were performed in the "Multi-DaphTrack" system. For esfenvalerate, carbofuran and abamectin experiments, the 5 controls were divided into two replicates of ISO water and 3 replicates of ISO water with 0.01% methanol (used to solubilize the respective compounds) to verify the absence of methanol induced effect on behavior. A randomization of location attribution was also performed for the different treatments with the constraint that two neighboring cells could not share the identical condition, i.e., vertically and horizontally. Each exposure cell was filled with 20 mL of the test solution, sealed with PARAFILM "M"® and maintained at 20 ± 0.5°C (a supplementary cell containing ISO water was placed near the platform and used to check the temperature over time), and ten neonates were carefully and randomly placed in each exposure cell.

Tests performed with the DaphToxI®

To compare behavioral trends from the "Multi-DaphTrack" system to a commercialized BEWS currently used in biomonitoring programs, one control and two replicates of a similar concentration tested in the "Multi-DaphTrack" system were tested in parallel for each compound in the DaphToxI® system (Bbe® Moldaenke, Kiel, Germany). Ten neonates were placed in each of the 3 exposure cells (25 mL) in different DaphToxI® systems. The experiment was performed under flow-through conditions at 33 mL/min and 20 ± 1°C, in which daphnids were first acclimated with an ISO water solution for 2 hours. The analysis of the behavior started at the beginning of the chemical exposure by replacing the ISO water with the test solution in a closed circuit after complete ISO water evacuation (the time was previously estimated at 3 minutes by a colorimetric measurement). To be

consistent between the immobilization test and the test in the "Multi-DaphTrack" system, the experiments in the DaphToxI® were conducted without a food supply.

Statistical analyses

For the acute toxicity test, concentration-response curves for immobilization were modeled using the Hill model in the Regtox© macro in Microsoft Excel. Effect concentrations (ECx) and their confidence intervals were estimated using the non-parametric "Bootstrap" method. As the behavioral data provided by the "Multi-DaphTrack" system are more complex and time-dependent, all further statistical analyses were performed with customized scripts in the statistical software R (R 3.0.1). To reduce the signal's noise and to allow for the comparison between different concentrations, the average speeds per condition and per hour together with the variability were calculated. After verifying the compliance of the variance homogeneity and the normal distribution of the data, a standard ANOVA model was performed with a simple Student test by independently comparing each concentration to the control test (p=0.01) for each hour. Dunnett's post hoc test (multiple comparisons) was performed to compare results from each treatment group with the corresponding control group. However, this latter test requires 48 h-averaged results and is too restrictive by not accounting for the variability of behavior over time. A Bonferroni correction can also be applied to counteract the problem of multiple comparisons and to control for the family-wise error rate. Similar to Dunnett's test, the Bonferroni correction was too restrictive to account for the variability in behavior. Therefore, we independently employed the T-test to compare each concentration to the control test (p=0.01) for each hour, which resulted in a total error rate of 29 minutes for the 48-h test (total error rate is calculated by multiplying the p-value with the exposure time). Due to the test capacity constraint of the DaphToxI® system, only one control was performed in each experiment. For comparison of results, 12 controls were combined and considered as control reference. A linear mixed effect model was therefore applied to account for the day-to-day variability in the *Daphnia magna*'s average speed in the controls; the variability in speed was considered a random effect. This model was used to independently estimate the (fixed) effect of exposure with respect to the control condition for each hour.

RESULTS

Acute toxicity tests

Experimental results of the standard immobilization acute test

The results of the experimental and QSARs-predicted 48-hour EC_{50} of *Daphnia magna* immobilization for the different tested compounds are presented in table 2. Overall, the experimental EC_{50} (48 h) values of the different substances agree with previous acute toxicity data collected in peer-

reviewed literature or on-line databases. As it is recommended in the OECD guideline 202, the pH stayed stable and the oxygen level was consistently > 3 mg/L at the end of the experiment and no differences between controls with and without 0.01% methanol were observed during the experiment, i.e., no immobility, no noticeable sign of stress, no discoloration. Standard immobilization tests (24h) with the reference chemical $K_2Cr_2O_7$ were also performed every month during the experiment period and the obtained values (EC_{50}) were in the recommended acceptable range and stable over time. *Daphnia magna* was shown to be highly tolerant to organic solvents (isopropanol, ethanol) and quite tolerant to caffeine and imidacloprid. *Daphnia magna* was also found to be moderately sensitive to sertraline and copper sulfate and more sensitive to fipronil and carbofuran. Finally, daphnids were found to be highly sensitive to trichlorfon, esfenvalerate, cypermethrin, abamectin.

Substance	Mode of action	Log K_{ow} (EPI)	EC_{50}, (48 h) (mg/L)	$_{exp}EC_{50,baseline}$ (48 h) (mg/L)	Excess toxicity ($LogT_e$)	$EC_{50,exp}$ (48 h) literature (mg/L)
Isopropanol	Narcotic	0.28	10100	929	-1.0	226; 630[a]
Ethanol	Narcotic	-0.14	7640	1580	-0.7	9248[a]
Caffeine	Adenosine receptor antagonist	0.16	177.8	3770	1.3	182[b]
Imidacloprid	Nicotinic acetylcholine receptor agonist	0.56	93.88	2320	1.4	56.6[c]
Sertraline	Selective serotonin reuptake inhibitor	5.29	0.560	0.352	-0.2	0.920[d]
Copper sulfate	Multiple	-	0.1754	-	-	0.183[e]
Fipronil	GABA receptor antagonist	6.64	0.0348	0.039	0.0	0.088; 0.190[a]
Carbofuran	Reversible acetylcholine esterase inhibitor	2.30	0.0182	74.1	3.6	0.020; 0.018[f]
Esfenvalerate	Modulator of sodium channels	6.76	0.00089	0.030	1.5	0.0009[g]
Cypermethrin	Modulator of sodium channels	6.38	0.00031	0.061	2.3	0.00042[a]
Abamectin	GABA receptor agonist	4.00	0.00027	5.44	4.3	0.00034[h]; 0.00025[a]
Trichlorfon	Acetylcholine esterase inhibitor	0.42	0.00021	3.05	4.2	0.00029[i]

Table 2: Results of 48 h *Daphnia magna* acute toxicity of the twelve selected compounds (predicted and experimental EC50 of the immobilization standard tests) according to their mode of action in comparison with values from the literature (a: (U.S. Environmental Protection Agency 2013), b: (OECD SIDS 2003), c: (Tišler et al. 2009), d: (Christensen et al. 2007), e: (Arambašić et al. 1995), f: (Fernández-Alba et al. 2002, Hernando et al. 2005), g: (Lewandowska 2004), h: (Tisler and Erzen 2006), i: (Coelho et al. 2011)).

Predicted $EC_{50,baseline}$ values ranged from 0.030 mg/L (esfenvalerate) to 3770 mg/L (caffeine). The predicted toxicity of ethanol and isopropanol slightly overestimated the acute toxicity compared to our experimental results. In contrast to the predicted $EC_{50,baseline}$ values, ethanol showed a higher experimental $EC_{50,exp}$ compared to isopropanol. Nevertheless, for both solvents, the excess toxicity, $LogT_e$, was in the range of baseline toxicity ($-1<LogT_e<1$). Despite the fact that excess toxicity is supposed to be driven by specific mode of action chemicals, predicted $EC_{50,baseline}$ values for the specifically acting compounds sertraline and fipronil were near their $EC_{50,exp}$ values. Consequently, these two compounds did not show excess toxicity ($-1<LogT_e<1$). However, all other specifically acting compounds (i.e., caffeine, imidacloprid, carbofuran, esfenvalerate, cypermethrin, abamectin and trichlorfon) exhibited an excess toxicity compared to baseline toxicity ($LogT_e>1$). Carbofuran,

trichlorfon, and abamectin showed a high excess toxicity when their $EC_{50,exp}$ values were 4.1 x 10^3, 1.4 x 10^4 and 2 x 10^4 times more toxic than predicted from the baseline toxicity, respectively.

Behavioral endpoint results

Tests performed with the "Multi-DaphTrack" system

All time courses for the swimming speed for the controls and the chemical-exposed *Daphnia magna* are available in the supplemental data file. Representative results are shown in Figure 22. Narcotics induced an intense increase of the swimming speed from the first hour of the experiment followed by a gradual decrease of the swimming speed (Figure 22 A and B). A marked and significant increase in swimming speed ($p<0.01$) occurred from the first hour of isopropanol exposure and extended for 21 and 30 hours at the concentrations of 1.3 and 2.4 g/L, respectively (far below the EC_5 at 48 h of the acute test), and before that, the average speed went back to control levels for both concentrations. The highest increase in the swimming speed (+159% above the control level, $p<0.01$) was observed for the concentration of 4.3 g/L (near the EC_5 at 48 h) of isopropanol during the first hour of exposure and lasted for 23 hours. For the two highest tested concentrations of isopropanol, a marked slowdown was observed from 10 hours of exposure at 7.7 g/L (near EC_{20} at 48 h) and from the first hour of exposure at 13.9 g/L (above the EC_{70} at 48 h); high inactivity occurred until the end of the experiment. Ethanol induced a smaller speed increase compared to isopropanol (highest increase 42% above the control level for the concentration 4.8 g/L (near the EC_{10} at 48 h, $p<0.01$)), but the effects globally followed the same trends as for isopropanol exposure (i.e., an increase followed by a gradual decrease of the swimming speed and a marked slowdown close to inactivity for the highest concentrations).

Toxicants with specific modes of action induced a slight but significant speed increase during the time course depending on their mode of action. Trichlorfon showed very late effects, whereas copper sulfate, sertraline and imidacloprid induced delayed effects on the swimming speed compared to the control level. The time courses of copper sulfate and trichlorfon exposure are depicted in Figure 22 (C and D, respectively). Copper sulfate exposure induced significant increases ($p<0.01$) from 5 to 11 hours for all tested concentrations. However, these increases were subtle, and the highest increase did not exceed +30% above the control level at 8 hours of exposure to 191.3 µg/L of copper sulfate, e.g., above the EC_{50} at 48 h. A significant decrease in the swimming speed was observed from 16 hours until the end of the experiment for the highest concentration of 363 µg/L (above the EC_{70} at 48 h). The results of the swimming speed over time of daphnia neonates exposed to different concentrations of trichlorfon did not show these marked changes in the swimming speed. The lowest concentration of trichlorfon displayed a slight but significant effect (+9% of speed increase compared to controls, ($p<0.01$)) and was 0.082 µg/L, e.g., above the EC_{20} at 48 h. A significant increase in the

swimming speed appeared later (from 26 hours) and did not exceed +39% above the control level ($p<0.01$) for the highest tested concentration of 0.336 µg/L (near the EC_{70} at 48 h).

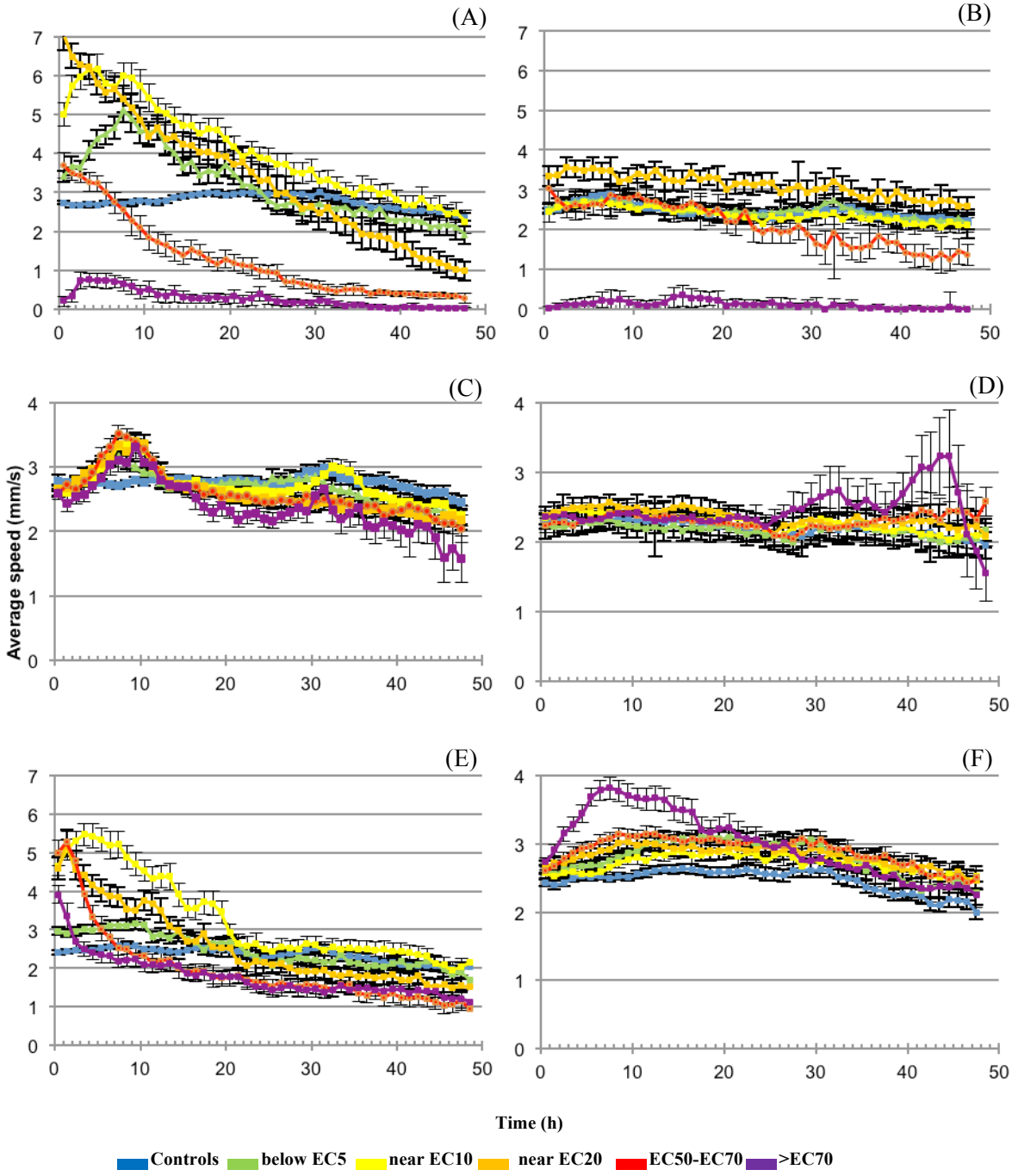

Figure 22: Average swimming speed ± standard error (per hour) of *Daphnia magna* neonates exposed to several concentrations of (A) isopropanol, (B) ethanol, (C) copper sulfate, (D) trichlorfon, (E) esfenvalerate and (F) fipronil for 48 h in the multi-cell exposure system.

The majority of the toxicants with specific modes of action (except trichlorfon, sertraline and imidacloprid) induced rapid and significant effects on the swimming speed. As illustrative examples, results obtained with esfenvalerate and fipronil are shown in Figure 22 E and F, respectively. Esfenvalerate induced a significant increase in the swimming speed for all tested concentrations from the first hour of the experiment. A slight but significant increase in swimming speed (+23% relative to

the controls, $p<0.01$) was observed for the lowest test concentration at 0.14 µg/L (below the EC_5 at 48 h) after the first hour of exposure and lasted for 16 hours. The swimming speed then returned to the control level. The most pronounced increase in swimming speed was observed at 0.35 µg/L (near the EC_{10} at 48 h), with the maximum speed occurring at 4 h (+123% relative to the controls, $p<0.01$). This exposure concentration caused an excitation in the daphnia for 21 hours before the swimming speed returned to the control level (the longest excitation effect duration observed in the experiment). Fipronil induced a small but significant increase in speed at the lowest tested concentrations of 1.4 µg/L (EC_5 at 48 h) to the highest concentration as soon as the first hour of exposure. The highest increase (+52% above the controls level, $p<0.01$) was observed after 8 hours of exposure for the highest test concentration.

Comparison of results between two behavioral analysis systems

In the DaphToxI® system, an average swimming speed of 3.65±0.13 mm/s was observed for control conditions over a period of 48 h; daphnids swam 1.5 times faster in the DaphToxI® system than in the "Multi-DaphTrack" system (2.35±0.18 mm/s) (Figure 23 A), which is most likely because of the stimulation of the imposed water flow in the flow-through system. A potential impact of the slightly different density in the two systems is unlikely as a prior study showed no influence of daphnia density on the average speed in the relevant density range (Chevalier et al. 2014). Whereas the swimming speed slightly decreased over time in the "Multi-DaphTrack" system, the average swimming speed remained stable in the DaphToxI® system. A time course of the average swimming speed of the control and representative results of chemical exposure of *Daphnia magna* neonates in the DaphToxI® is shown in Figure 23 B. Significant increases in swimming speed were observed for exposures (between EC_5 and EC_{20}) to 4.3 g/L of ethanol ($p<0.01$), 0.35 µg/L of esfenvalerate ($p<0.01$), 0.04 µg/L abamectin ($p<0.05$) and 0.26 µg/L of sertraline ($p<0.05$). For the other compounds, the tested concentrations were too low to induce a significant effect on the swimming speed. Fipronil exposure in the DaphToxI® system did not show significant results but similar speed increase trends were observed in the DaphToxI® system compared to the "Multi-DaphTrack" system in the first 6 h of exposure. Unexpectedly, the results in response to imidacloprid showed a significant decrease in the swimming speed from 2 to 9 h of exposure at the concentration of 85 mg/L (between the EC_{20} and the EC_{50} at 48 h), whereas a significant increase was observed in the "Multi-DaphTrack" system.

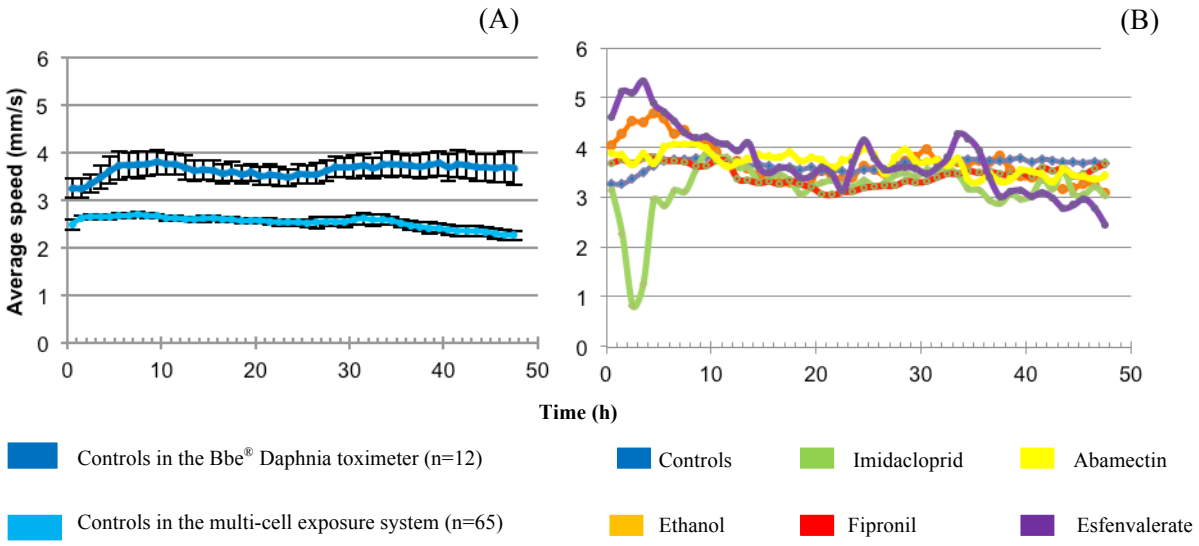

Figure 23: (A) Average swimming speed with standard error (per hour) of *Daphnia magna* neonates exposed to controls in the DaphToxI® system and in the multi-cell exposure system and (B) average swimming speed (per hour) of *Daphnia magna* exposed to controls and one concentration (nearby EC_{10}) of ethanol, esfenvalerate, imidacloprid, fipronil and abamectin for 48 h in the Bbe® DaphToxI® system.

Sensitivity, rapidity and duration of the behavioral response

To compare the sensitivity of the swimming speed to the standard immobilization endpoint, we calculated the ratio of the EC_{50} (48 h) of the standard immobilization acute toxicity test to the lowest behavioral effect threshold. The sensitivity ratio, effect onset and duration of response of the different tested chemicals are summarized in Table 3. Except for trichlorfon, the lowest behavioral threshold is more sensitive for all tested compounds compared to the $EC_{50,exp}$ (48 h) of the acute immobilization standard test. The highest sensitivity of the swimming speed compared to the standard immobilization endpoint was found for fipronil, with a sensitivity ratio equal to 25. By contrast, behavioral endpoints were only slightly more sensitive for carbofuran, ethanol and imidacloprid, with a sensitivity ratio ranging from 1.6 to 1.1. For most of the compounds tested, behavioral responses were observed earlier (i.e., from the first hour of exposure) in comparison to the immobilization assay, except for copper sulfate, sertraline and imidacloprid, which showed delayed effects, and trichlorfon, which showed late effects. Interestingly, sertraline and fipronil, which were selected for their modes of action (a selective serotonin reuptake inhibitor and GABA receptor antagonist, respectively) and exerted acute toxicity in the range of the baseline toxicity (Table 2), showed the highest sensitivity ratios for behavioral responses. This observation confirms the specificity of these compounds in their toxicity in daphnia and highlights the fact that certain specific effects may be overlooked when focusing on immobilization only.

Substances	Acute test	Multi-cell exposure system					DaphToxI
	$EC_{50,exp}$ (48 h) (mg/L)	Lowest concentration threshold (mg/L)	Time effect onset (h)	Duration (h)	Sensitivity ratio	Toxicokinetic index (mg.h/L)	Significant effects * (p<0.05) **(p<0.01)
Fipronil	**0.0348**	**0.0014**	**1**	**33**	**24.9**	**0.0014**	-
Sertraline	**0.560**	**0.030**	**5**	**3**	**18.7**	**0.15**	*
Isopropanol	**10100**	**1300**	**1**	**23**	**7.8**	**1300**	-
Cypermethrin	**0.00031**	**0.00004**	**1**	**7**	**7.8**	**0.00004**	-
Caffeine	**177.8**	**25**	**1**	**20**	**7.1**	**25**	-
Esfenvalerate	**0.00089**	**0.00014**	**1**	**16**	**6.4**	**0.00014**	**
Copper sulfate	**0.1754**	**0.0279**	**5**	**3**	**6.3**	**0.1395**	-
Abamectin	**0.00027**	**0.000043**	**2**	**29**	**6.3**	**0.000086**	*
Carbofuran	**0.0182**	**0.0107**	**2**	**27**	**1.7**	**0.0214**	-
Ethanol	**7640**	**4800**	**1**	**48**	**1.6**	**4800**	**
Imidacloprid	**93.88**	**85**	**10**	**5**	**1.1**	**850**	**
Trichlorfon	**0.00021**	**0.000336**	**39**	**7**	**0.6**	**0.013104**	-

Table 3: Sensitivity, time onset and duration of responses of *Daphnia magna* neonates to chemicals with different modes of action.

DISCUSSION

Contrasting profiles of swimming speeds according to the chemical tested

Two behavioral syndromes of stress were identified following the exposure of daphnia neonates: an increased average speed or a decrease of the average speed relative to the control level in a concentration-dependent manner. Contrasting profiles of swimming speed effects were observed among tested compounds for the time of response, the duration and the intensity of effects on the swimming speed. For instance, an intense and rapid increase in the swimming speed followed by a gradual decrease in the swimming speed was induced after exposure to narcotics. In contrast, the effects of specific or multiple modes of action compounds on the swimming speed were more heterogeneous and characterized either by a steep or a slight increase followed by a rapid slowdown back to or below the control level. Besides, the time of onset of effect also differed among specifically acting compounds, with a rapid, delayed and late onset of effects.

Toxicokinetic processes are likely involved in the time of effect onset because the toxicant must go through the epidermal tissue to reach the target site (e.g., nervous system for neurotoxin) and exert adverse effects. For instance, immediate responses were observed for half of the specifically acting chemicals (i.e., esfenvalerate, fipronil, cypermethrin, abamectin, caffeine and carbofuran) and may result from a fast absorption and tissue distribution of the compounds. On the other hand, delayed behavioral effects, as induced by sertraline exposure, may likely result from chemical uptake and distribution in daphnids and to efficient defense mechanism activation. However, sertraline is an emerging pollutant whose ecotoxicity is still poorly documented. Behavioral effects induced by copper

sulfate exposure were also delayed and transient: a significant increase of the mean speed ($p<0.01$) was observed from 5 to 11 hours at all tested concentrations. This observed delay for behavioral effects might be explained by the time required for metal uptake, which depends on the speciation of copper. The relatively rapid recovery of daphnia exposed to copper sulfate may be related to protective mechanisms, such as intracellular metal sequestration through metal-binding proteins (i.e., metallothioneins), or detoxification mechanisms (e.g., elimination through the sodium, potassium or calcium channels of the cellular membranes mediated by specific transport systems such as cation ATPases). Besides, a significant decrease of the swimming speed was observed at the highest concentration and may indicate that protective mechanisms are saturated, resulting in deleterious effects. These results are in accordance with previous studies (Jeon et al. 2008), which reported hyperactivity at 7 hours for a concentration of 50 µg/L. Imidacloprid showed an intermediate delay in the behavioral responses. However, a previous study reported a rapid increase of cumulative distance swum (which is similar to average speed) by *Daphnia pulex* at higher concentrations (Zein et al. 2013). Finally, trichlorfon exposure produced unexpected results: observed effects were delayed and occurred at concentrations similar to the time of immobilization effects. On the other hand, the two independent variables, i.e., time and concentration, can be combined by calculating a toxicokinetic index (see table 3). All substances showed a toxicokinetic index inferior to 1 mg.h/L indicating that most of the tested substances can induce significant behavioral effect very rapidly. In this study, the onset of effects of toxicants was observed to be rapid or delayed according to the different tested modes of action, highlighting the importance of following effects over time to aid in the determination of compounds with transient or persistent behavioral effects.

Do compounds sharing the same mode of action show similar behavioral effects?

After establishing a library of sub-lethal behavioral responses to a series of defined modes of actions, this approach could be used as a baseline tool for identifying the chemical present based on observed behavioral profiles in water quality monitoring programs. However, for this outcome to be achieved, dissimilar chemical modes of action must be determined to elicit specific and distinct behavioral response profiles that can predict toxicity. Both isopropanol and ethanol, acting through narcosis, showed similar time course effects on the average speed (i.e., an intense increase followed by a gradual decrease). For narcosis, lipophilic compound uptake is governed by partitioning into biological membranes (Escher and Hermens 2002). No active transport process or metabolism is involved, which can explain the immediate behavioral response that was observed (time for daphnia to reach equilibrium with the surrounding aqueous phase). The “narcotic” compounds surprisingly induced long-lasting behavioral excitations; “narcosis” is commonly related to reversible anesthetic effects, i.e., depressed locomotion (hypoactivity syndrome), as noted by Drummond and Russom (1990). Contrasted profiles of swimming speed effects were observed for specifically acting chemicals. The intrinsic potency of specifically acting compounds may be dependent on their affinity to, or type

of interaction with the target receptor (Escher and Hermens 2002). Esfenvalerate and cypermethrin share an identical mode of action as a modulator of sodium channels, and both exhibited a rapid effect on the swimming speed. Although fipronil and abamectin have dissimilar modes of action (opposite mechanisms on GABA receptors, i.e., antagonist and agonist, respectively), both induced similarly rapid behavioral effects. However, among compounds with a similar mode of action, differences in toxicity potency, time to effect onset and duration of effects may be observed.

Our starting hypothesis was that compounds with similar modes of action could exhibit similar behavioral effect profiles. Esfenvalerate and cypermethrin, which share similar effect profiles, could corroborate this hypothesis. Unexpectedly, carbofuran and trichlorfon exposures induced different effect patterns, whereas these two compounds share a similar mode of action, i.e., AchE inhibition (Pope et al. 2005). Carbofuran exposure showed rapid but slight effects on the swimming speed, which went back to the control level at the end of the exposure. This pattern reflected a rapid recovery of the AchE enzyme (graph available in the supporting information). Trichlorfon exposure induced more significant but late effects on the swimming speed. These differences might be explained by a difference in the toxicokinetic behavior of the compounds because of subtle differences in the mechanism of action, i.e., phosphorylation and carbamylation of the AchE enzyme by trichlorfon and carbofuran, respectively, or carboxylesterase inhibition (Barata et al. 2004). Distinct behavioral profiles can be distinguished based upon the mode of action. However, in some case, the identification of the mode of action based on the behavioral effects profiles can be biased. Indeed, chemicals with dissimilar modes of action can show similar behavioral effects, e.g., isopropanol and esfenvalerate have similar effect profiles but dissimilar modes of action. Furthermore, $EC_{50,exp}$ (48 h) values for specifically acting compounds with high hydrophobicity (i.e., $\log K_{ow}>5$), e.g., cypermethrin, and esfenvalerate, tend to deviate less from baseline toxicity than hydrophilic compounds. Despite sertraline and fipronil being well recognized as specifically acting compounds, their excess toxicity occurs between $-1<\log T_e<1$, indicating a baseline toxicity. In this case, the "dominant" mode of action for this compound may act through narcosis instead of neurotoxicity. When the log K_{ow} is high, the toxicity of specifically acting compounds may be driven by hydrophobicity because of a high adsorption of the compound in all membranes. Furthermore, no distinct behavioral profiles could be drawn from the chemical mode of action based on the calculated toxicokinetic index values. Our results emphasize the fact that interpretations of effect profiles may not always be straightforward, and specific interactions with target-sites must be considered.

Presumably, the increase in the swimming speed is a stress response designed to escape the contaminated area. The escape behavior is also called avoidance and has been reported in the literature for crustaceans (De Lange et al. 2006, Eriksson Wiklund et al. 2006, Hellou et al. 2005, Kravitz et al. 1999). The increase of the swimming speed is the most frequently reported behavioral effect and is used as an indication of early stress in *Daphnia magna* when exposed to chemicals (e.g., sublethal OPs

exposure (Ren et al. 2009 (b)) or metal exposure (Cd) (Untersteiner et al. 2005, Wolf et al. 1998)). In our study, the swimming speed was the most sensitive parameter compared to other parameters (number of active organisms and changes in path angle). Furthermore, the average speed increase, which may indicate avoidance, was earlier and showed much more contrasted profiles than the average speed decrease. Therefore, the average speed appears to be a suitable stress indicator.

Sensitivity and utility of the new "Multi-DaphTrack" system

In the present study, we attempted to systematically analyze a large set of chemicals with different modes of action to evaluate the sensitivity and onset of a behavioral parameter in the newly developed "Multi-DaphTrack" system. Generally, changes in swimming speed provide early and sensitive information compared to standard test endpoints. With the exception of trichlorfon, the average speed endpoint was shown to be more sensitive than the immobilization parameter of the acute standard test for all tested chemicals. Instead of waiting 48 hours to receive toxicity results, behavioral tests provide information within the first hour of exposure. Furthermore, behavioral effects occurred at concentrations well below the acute toxicity level (the lowest significant behavioral threshold is below the EC_{10} (48 h) for most of the compounds tested). Similar rapid increase of the average swimming speed has been observed in copepods under toxicant exposure and show that our results can be generalized to other zooplankton groups (Michalec et al. 2013). Behavioral results obtained in the "Multi-DaphTrack" system can be also compared with chronic toxicity data (reproduction test, 21 days) available in literature and on-line databases (see additional support (d)). Overall, the chronic toxicity assay tends to be more sensitive than the behavioral assay. This result is obviously due to a longer exposure time over one generation cycle in the chronic assay in comparison to 48h for our behavioral assay. Nevertheless, behavioral test still represents great advantages compared to the standard chronic test since behavioral change thresholds are not so far from chronic toxicity thresholds and behavioral tests are less time consuming. To date, only anecdotal behavioral effect thresholds have been reported for isolated chemicals in specific daphnia behavioral analyses in laboratories. In general, as the swimming speed parameter showed higher or similar sensitivity compared to the acute toxicity endpoint, this parameter appears to be well suited to detect pollution in water quality monitoring or effluent toxicity assessment. The experimental designs of the new "Multi-DaphTrack" system involved several replicates, with ten individuals per replicate, and well-controlled experimental conditions to minimize the considerable variability in behavioral data. Furthermore, most of the behavioral responses (i.e., speed increase) were observed shortly after the beginning of exposure. Nevertheless, accurately extrapolating from behavioral effects measured in highly structured and controlled laboratory experiments to complex and readily changing field situations is difficult.

In the environment, toxicants commonly occur in mixtures rather than as individual pollutants, and combined effects of toxicant mixtures and natural stressors are typically observed (Holmstrup et al. 2010). One solution to the problems posed by mixtures would be to evaluate the effect of such

environmental mixtures on the behavior of living organisms; the organisms could be exposed to mixtures in the "Multi-DaphTrack" system under controlled conditions. Another solution would be the use of *in situ* online BEWSs that directly expose organisms to field water. We wanted to compare the results of the "Multi-DaphTrack" system to the currently used BEWS, namely, DaphToxI®. The "Multi-DaphTrack" system was more sensitive than DaphToxI® because few significant results for the swimming speed were obtained with the latter system (only three compounds induced significant effects on the swimming speed in DaphToxI®). This sensitivity difference could be explained both by the higher variability in behavioral responses under flow-through conditions in the DaphToxI® system and by the low number of replicates (n=2) preventing significant differences despite the observed effect trends. However, the selection of rather low-test concentrations in the DaphToxI® system (because of the need to perform all behavioral tests for each compound in parallel) can also be criticized because the low concentrations overestimated the sensitivity of behavioral endpoints. While a significant increase was observed in the "Multi-DaphTrack" system, the imidacloprid exposure results in the DaphToxI® system showed a significant decrease in the average speed. This contrasting result may be because of the influence of the flow-through condition in the DaphToxI® system and to the fact that imidacloprid is known to cause disorientation. However, this study represents initial exploratory research aiming to unravel behavioral effects in monitoring systems. Although not all of the selected concentrations of compounds tested in the DaphToxI® system produced significant effects, similar trends were observed between the two test systems. Future assessments must be performed with higher concentrations and/or concentration ranges. Daphnia BEWSs (such as DaphToxI® and MFB®) have been found to be sensitive to acute aquatic pollution, and their use is recommended in monitoring accidental contamination (Ren et al. 2009 (a), Ren et al. 2009 (b), Zeng et al. 2012). *In situ* BEWSs set up directly in the field may provide an initial insight to assist in the evaluation of the effects of pollutants in an environmental context. In contrast to chemical monitoring, which typically focuses on the quantification of a predefined target compound, biological toxicity assessment accounts for the totality of bioavailable contaminants as well as potential mixture effects (European commission 2014).

To conclude, the new "Multi-DaphTrack" system provided early and sensitive responses to sub-lethal toxic stresses. Therefore, this system could be used as an alternative or complement to the current acute standard test or chemical analysis for the predictive evaluation of toxic effects in effluent toxicity assessments. For the purpose of surface water or effluent monitoring, the time-consuming acute immobilization test can potentially be replaced by more sensitive and early tests. However, the behavioral stress measured in BEWSs (such as DaphToxI®) can be used as retrospective screening tools for environmental mixtures of unknown contaminant compositions and as an early warning system of exposure and effects. Therefore, BEWSs can be used as a valuable tool for exposure and effect evaluation in water monitoring, and supplementary analyses can be performed on the "Multi-DaphTrack" system when a more thorough understanding of the concentration dependence of effect

patterns is required. In this case, only 10 hours analysis would be necessary, since most tested compounds with different modes of actions responded in the first 10 hours.

ACKNOWLEDGMENT

This work was supported by EDF in collaboration with Bordeaux1 University and INERIS, France. We thank S. Maestri, for technical assistance during the experiment and D. Chadaigne and U. Azmi for the help developing R-scripts for data treatment and statistical analysis.

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CHAPTER IV: SYNTHESIS AND DISCUSSION

1. BEHAVIORAL ANALYSIS IN *DAPHNIA MAGNA*

1.1 Analysis of the basal swimming activity of *Daphnia magna*

The biological behavior variability is quite high and this variability may prevent the detection of significant differences from control levels. By increasing the number of replicates in our multi-cell exposure system, it was possible to decrease the variability of the average speed per hour and hence facilitating the detection of significant effects induced by toxicant exposure at low concentrations. Our results showed that the individual average speed of isolated Daphnia was significantly lower than average speed of a daphnia group. Daphnia behavioral systems with high-test capacity developed so far are mainly focused on the individual tracking. Nevertheless, in the field conditions, daphnids usually stay in groups (Dieter 2005) and it may be therefore more ecologically relevant to track group of daphnids instead of individuals for toxicity prediction. The "Multi-DaphTrack" system is, to our knowledge, the behavioral analysis system with **the highest test capacity** for **groups of daphnids** with **exposure condition close to the standard acute immobilization test**. In this way, experiments with several concentrations and replicates can be performed simultaneously and Daphnia behavior can be tracked for a period as long as 48 hours.

The basal activity of Daphnids exposed in static control conditions in the "Multi-DaphTrack" system is quite homogeneous over time. The exposure conditions in the "Multi-DaphTrack" system, i.e., absence of light, food and the size and age calibration, have significantly contributed to decrease of the variability of daphnia behavior over time and prevent the expression of daphnia's biological cycle. The average speed of Daphnia groups in the "Multi-DaphTrack" system (48 h-average speed of 2.55 ± 0.11 mm/s) is within the range of previous studies for groups of Daphnia of less than 24 hours age: average speed of ≈3.1 mm/s for *Daphnia magna* in Untersteiner et al. (2003) and 2.7 mm/s for *Daphnia pulex* in Artells et al. (2013). A bigger average speed (>5 mm/s) was observed for the species *Daphnia similis* aged of less than 24 hours age (Artells et al. 2013). It was also noted that the flow-through condition exposure increases the *Daphnia magna* average speed (3.65 ± 0.13 mm/s obtained in the DaphTox®), hence the water flow appears to stimulate daphnia swimming. According to the natural water flow (river with continuous flow or small ponds with stagnant water), the average speed may greatly vary in the real field conditions.

1.2 Behavioral responses induced by chemical exposure in the "Multi-DaphTrack" system

1.2.1 Selection of the average speed as parameter for toxicity assessment

Basically, different behavioral syndromes of stress were identified following chemical exposure of daphnia neonates: an **increased average speed**, a **decreased average speed** and a **decrease of the number of active organisms** relative to control level in a concentration dependent manner. For all tested compounds, the average speed increase was the earliest and most sensitive

observed effect. Whatever the considered chemical, the increased average speed was much more contrasted over time (in the effect onset, duration and intensity) than the decreased average speed or the decrease of the number of active organisms. The average speed appears to provide the most consistent responses. That is why; the study of chemical induced behavioral effects was mainly **focused on the increase trends of average speed**.

1.2.2 Sensitivity of the average speed parameter

The average speed parameter observed in the "Multi-DaphTrack" system was compared to the immobilization parameter of the standard acute test. As expected, the values of the lowest observed behavioral effect concentration (i.e., increase of the average speed) were generally much lower compared to the EC_{50} (48 h) of the acute immobilization standard test for all tested chemicals, except for trichlorfon (Figure 24).

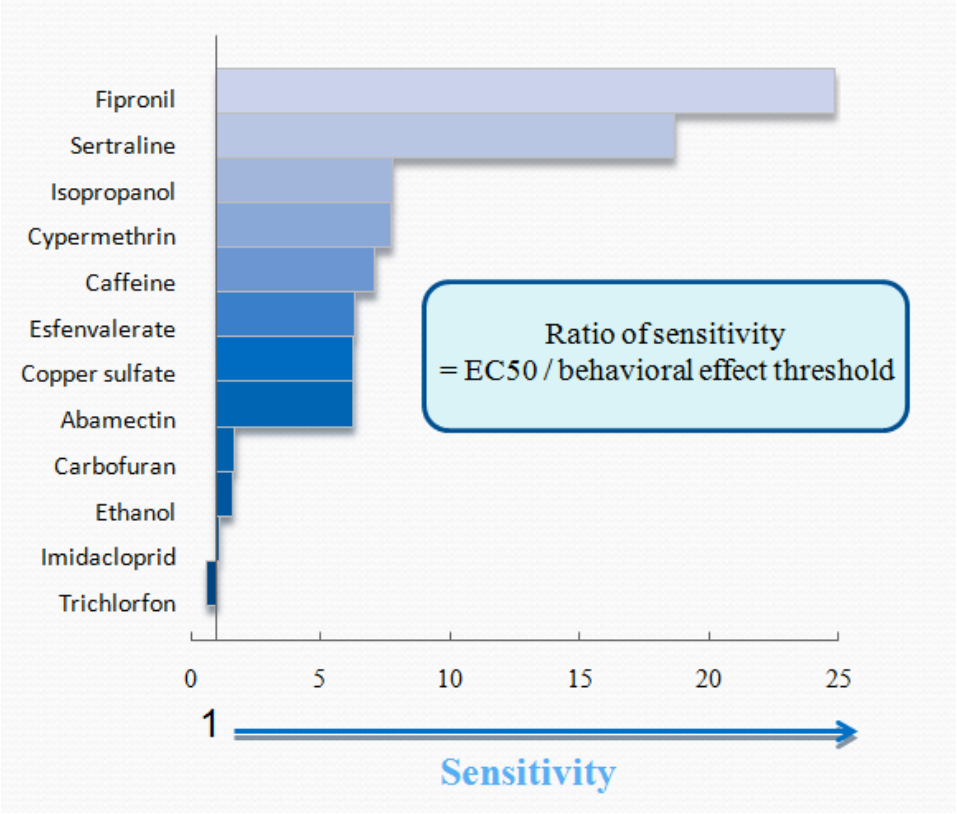


Figure 24: Sensitivity ratio obtained by dividing the EC_{50} of the acute immobilization assay with the lowest behavioral effect threshold (behavioral LOEC) of the "Multi-DaphTrack".

The lowest significant behavioral threshold (behavioral LOEC) is below or equal to the EC_5 (48 h) for fipronil, sertraline, isopropanol, caffeine, esfenvalerate sulfate copper, carbofuran and nearby the EC_{10} (48 h) for cypermethrin, abamectin and ethanol. The lowest behavioral effect concentration LOEC and the no observed behavioral effect concentration NOEC have been determined for each compound and are listed in the Table 4. NOEC determination was not possible for most of tested compounds because

significant effects were observed at the lowest exposure concentration. However the determination of NOEC depends a lot on the selection of testing concentrations.

1.2.3 Earliness of the average speed increase effect

Early stress responses (i.e., increase of the average speed) were revealed under chemical exposure in the "Multi-DaphTrack" system (see Table 4) for most of the tested compounds (fipronil, isopropanol, cypermethrin, caffeine, esfenvalerate, ethanol, carbofuran and abamectin) at concentrations well below the EC_{50} (48 h). Copper sulfate, imidacloprid and sertraline showed intermediate effects, and latency was observed for trichlorfon. Except for trichlorfon, most of the tested compounds significantly induced early behavioral responses from the first hour of exposure and intermediary behavioral responses before the first 12 hours of exposure. These results showed that instead of waiting the 24 or 48 hours required for standard acute immobilization assay, behavioral tests provide information in few hours of exposure. This study showed that the behavioral effect assessment over time could provide useful and early signal of chemical contamination.

Table 4: Synthetic acute and sub-acute toxicity data for *Daphnia magna* neonates exposed to different chemicals with new results.

Substance	Acute test	"Multi-DaphTrack" system			
	$EC_{50,exp}$ (48 h) (mg/L)	Behavioral NOECs (mg/L)	Behavioral LOECs (mg/L)		
			Threshold	Onset-Duration	Intensity of increase Mean % [min-max]
Isopropanol	10100	**x**	**<1300**	1-21	**45 [16-88]**
Ethanol	7640	**1920**	**4800**	1-28	**30 [17-42]**
Caffeine	177.8	**12.5**	**25**	1-17	**47 [21-61]**
Imidacloprid	93.88	**34**	**85**	10-5	**27 [23-33]**
Sertraline	0.560	**x**	**<0.03**	5-3	**6 [5-8]**
Copper sulfate	0.1754	**x**	**<0.028**	5-3	**7 [5-9]**
Fipronil	0.0348	**x**	**<0.001**	1-33	**14 [6-21]**
Carbofuran	0.0182	**x**	**<0.011**	2-27	**11 [6-21]**
Esfenvalerate	0.00089	**x**	**<0.0001**	1-16	**20 [13-25]**
Cypermethrin	0.00031	**0.00002**	**0.00004**	1-7	**7 [5-9]**
Abamectin	0.00027	**x**	**<0.00004**	2-29	**27 [8-43]**
Trichlorfon	0.00021	**x**	**0.00034**	39-17	**19 [12-26]**

1.2.4 Intensity of effects on the average speed

For the lowest behavioral threshold obtained in the "Multi-DaphTrack" system, the intensity of the increased average speed was calculated relative to control level, averaged over the effect duration and expressed in percentage (see Table 4). The increase intensity of the average speed was contrasted between tested chemicals. The highest increase intensities were observed for isopropanol, ethanol, caffeine, imidacloprid and abamectin with an average speed around 30% above the control level. Fipronil, carbofuran, esfenvalerate and trichlorfon induced intermediate increases (around 20% above the control levels). Finally, slight increases of the average speed were observed for sertraline, copper sulfate and cypermethrin with only +7 % of intensity increase above control level.

Higher concentrations of chemicals induced also increased average speed in a greater degree. For instance, isopropanol exposure induced an intense increase of +159% above the control levels when exposed to a concentration of 4.3 g/L (below the EC_5 at 48 h). A mean of +79% of increase of the average speed above the control level was also observed from 1 to 21 h of exposure to 0.352 µg/L of esfenvalerate (near the EC_{10} at 48 h) and reached a maximum of +123% at 4 h of exposure. The intensity of the increase effect on the average speed was also higher for concentrations above the EC_{50} or EC_{70} of fipronil (maximal increase of +50% above the control levels at 7 h of exposure).

1.2.5 Evolution of the average speed over time

It is not a current practice to monitor effects over time despite the fact that it is well known that the effects of chemical stressors change temporally and depend on exposure duration (Heckmann et al. 2010). Detection of effects over time may be useful in water quality assessment and that is why we decided to monitor behavioral effects over time in this thesis' work. Results from the different chemical exposures in the "Multi-DaphTrack" system showed that effects on the average speed vary over time. As it was previously described, two types of effects can be observed on the average speed, i.e., increase and decrease. Except for fipronil and abamectin, which did not induced decrease effects on the average speed, all the tested compounds induced an increase of the average speed at the beginning of the experiment and then a decrease of the average speed at the end of exposure. For instance, significant increases of the average speed were observed for daphnids exposed to 1.3, 2.4, 4.3 g/L (concentrations $<EC_5$ (48 h)) and 7.7 g/L (between the EC_{10}-EC_{20} (48 h)) of isopropanol at the beginning of the experiment and significant decreases of the average speed were observed later in the course of the exposure for the concentration of 4.3 g/L ($<EC_5$ (48 h)) and 7.7 g/L (between the EC_{10}-EC_{20} (48 h)). For the highest concentrations of 13 g/L ($>EC_{70}$ (48 h)), a significant decrease effect on the average speed was observed directly from the first hour until the end of exposure. On the other hand, after an observed increase effect, recovery may be observed depending on the concentration tested. For instance, significant increased average speeds were firstly measured for carbofuran

exposure and then, the average speed went back to the control level. These results might indicate that behavioral effects can be reversible and/or animals are able to adapt themselves to the exposure. Nevertheless, even if recovery may occur after an increased average speed, the transient increase effect on the average speed may affect the long-term survival of the species and in this particular case, the standard immobilization test would obviously underestimate the risk of carbofuran exposure.

For most of the tested compounds, a decrease of the average speed was observed at the end of the experiment for the highest concentrations tested. These results corroborates with the observed gradual decrease of the number of active organisms observed at the end of the experiment. Likewise, no decrease of average speed was observed following exposure of Daphnia to high concentrations of carbofuran and trichlorfon leading respectively to 50% or 70% of immobilization at 48 h. The immobilization may not necessarily be correlated to the decrease of the average speed. For instance, the average speed of the group of daphnids can stay stable despite that some individuals are already immobilized. The density dependence on the average speed was previously examined and it was concluded that there is no impact on the average speed for groups of 3, 5, 10 or 15 daphnids. In fact, mortality may occur for the most sensitive individuals but the average speed of the surviving daphnids may not be impacted. Finally, this study shows that the monitoring of behavioral effects over time provides useful information on chemical effects over time and thus toxicokinetic of chemicals.

1.3 Behavioral effects induced by chemical exposure in the DaphToxI®

1.3.1 Synthesis of results of the twelve substances tested in the DaphToxI®

Results of chemical exposure in the DaphToxI® showed significant increase on the average speed after exposure to 4.3 g/L of ethanol ($p<0.01$) (between EC_5 and EC_{20}), 0.35 µg/L of esfenvalerate ($p<0.01$), 0.04 µg/L abamectin ($p<0.05$) and 0.26 µg/L of sertraline ($p<0.05$). For the other compounds, the tested concentrations were too low to induce a significant effect on the average speed. Fipronil exposure in the DaphToxI® system did not show significant results but similar speed increase trends were however observed compared to the "Multi-DaphTrack" system in the first 6 h of exposure. Unexpectedly, the results in response to imidacloprid showed a significant decrease in the average speed from 2 to 9 h of exposure at the concentration of 85 mg/L (between the EC_{20} and the EC_{50} at 48 h), whereas a significant increase was observed in the "Multi-DaphTrack" system. Overall, our results indicate that the DaphToxI® system is less sensitive in comparison to the "Multi-DaphTrack" system. However, this fact can be explained by the flow-through condition, which leads to increase in variability in the average speed as observed in the control results from both systems in the **chapter II**. It can also be explained by the lower number of replicates allowed by the DaphToxI® system, which provides less robust data.

1.3.2 Comparison of the results obtained in the DaphToxI® with the literature

In our study, the normal functioning of the DaphToxI® was modified aiming to compare with the standard acute immobilization assay and the "Multi-DaphTrack" system conditions. Modifications included the feeding supply cut off and the alarm deactivation (i.e., only the average speed parameter was monitored). It is noteworthy that the DaphToxI® is originally designed to detect a deviation from a previous recorded behavior considered as "normal" or "reference" behavior. Several behavioral parameters are monitored (average speed, average height, distance between organisms, etc.) over time and a "toxic index" is calculated based on the deviation of monitored behavioral parameters. Each deviation is scored in the "toxic index" with weighted parameters and when the "toxic index" overpasses a fixed threshold, an alarm is triggered. However, our exploratory results can be still compared to the literature.

Table 5: Results of average speed effects obtained in the BEWs DaphToxI in comparison with other toxicity tests and literature. N.D.: No Data. (a: (Chancerelle et al. 2010) b: (Werth 2006) c: (Lewandowska 2004)).

Substance	Acute test	"Multi-DaphTrack" system	DaphToxI®		Literature DaphTox®
	EC_{50} (mg/L)	Behavioral LOECs (mg/L)	**Concentration (mg/L)**	**Significant effects * (p<0.05) **(p<0.01)**	**Alarm threshold (mg/L)**
Isopropanol	10100	<1300	**1980 (<EC_5)**	-	N.D
Ethanol	7640	4800	**4800 (EC_{10})**	**	N.D
Caffeine	177.8	25	**12.5 (EC_5)**	-	N.D
Imidacloprid	93.88	85	**85 (EC_{20}-EC_{50})**	**	N.D
Sertraline	0.560	0.03	**0.26 (EC_{20})**	*	N.D
Copper sulfate	0.1754	<0.028	**0.1 (EC_{20}-EC_{50})**	-	**0.100[a]**
Fipronil	0.0348	<0.001	**0.007 (EC_{20})**	-	N.D
Carbofuran	0.0182	<0.011	**0.0142 (EC_{20})**	-	**0.110[b]**
Esfenvalerate	0.00089	<0.0001	**0.00035 (EC_{10})**	**	**0.001[c]**
Cypermethrin	0.00031	0.00004	**0.00009 (EC_{20})**	-	**0.002[b]**
Abamectin	0.00027	<0.00004	**0.00004 (<EC_{10})**	*	N.D
Trichlorfon	0.00021	0.00034	**0.0001 (EC_{20})**	-	**0.001[c]**

For instance, a previous study with the DaphToxI® has reported behavioral effects induced by the exposure to the sodium channel inhibitor **esfenvalerate** and the acetylcholinesterase inhibitor **trichlorfon** (Lewandowska 2004). The experimental conditions of exposure were different: to reproduce a pollution peak that can occur in real field situation, a "Gaussian" contamination wave has

been performed with a pump. In this study, several concentrations and several wave durations (3, 24 and 48 hours) were tested with the two chemicals esfenvalerate and trichlorfon. Obviously, the highest tested concentrations induced the earliest alarm for the two substances. With a contamination wave of 48 hours, the lowest concentration of **trichlorfon** triggering an alarm was equal to 1 µg/L and occurred at the 25th hour of exposure (see Table 5**Erreur ! Source du renvoi introuvable.**). The behavioral parameters which contributed to the alarm were the average speed, speed classes and the number of active organisms. To observe an early signal, a concentration as high as 8 µg/L of trichlorfon was needed and in this case, the alarm triggering is mainly due to the death of daphnids. With a contamination wave of 48 hours, the lowest concentration inducing an alarm for **esfenvalerate** was also equal to 1 µg/L. However, the alarm was triggered at the beginning of exposure, well before the contamination wave maximum. Thus, the concentration inducing the alarm for esfenvalerate exposure was estimated between 0.2 and 0.4 µg/L, which is closed to our tested concentrations (0.35 µg/L). In this study, the alarm was mainly caused by the increase of the average speed, which corroborates with our results. Interestingly, the behavioral responses induced by trichlorfon exposure in Lewandowska study were delayed relative to the contamination wave while esfenvalerate exposure induced immediate effects on daphnia behavior. Although experimental exposure conditions and tested concentrations were different, this study shows similar pattern in the average speed effects compared to our results obtained with the DaphToxI®. The delay of behavioral response observed for trichlorfon compared to esfenvalerate, reinforces the hypothesis behavioral effect onset can occur at different times according to the studied chemical and that indirect effects on behavior may also occur.

The organophosphate chlorpyrifos, which shares the same mode of action with **trichlorfon** (i.e., AchE inhibitor), was also tested in the DaphToxI® by INERIS using contaminated water samples from mesocosms. Chlorpyrifos induced an alarm in the DaphToxI® after 9 hours of exposure to a concentration above the EC_{50} (48 h) (Chancerelle et al. 2010). An increase of the average speed was detected one hour prior the beginning of mortality and the alarm triggering. This result indicates that, as it was obseverd for trichlorfon, AchE inhibitors do not necessarily induce early behavioral effects and a delay in the behavioral responses may be observed. Daphnids aged of 78 hours and exposed to 100 µg/L of **copper sulfate,** exhibited immediate and transient behavioral changes, i.e., increase of the average speed, constant decrease of the height of swimming, increase of the distance between organisms and alarm induction. In our study, surprisingly, **no significant effects and no average speed increase trends** were observed for the same tested concentration of 100 µg/L of copper sulfate. This may be explained by the fact that daphnids were older and fed (2 factors that increase the average speed) when exposed to sulfate copper in the study of INERIS. Interestingly, however, the "height of swimming" parameter, as the "distance between organisms" parameter did both contribute in the alarm induction. Hence, these two parameters should be investigated in case of sulfate copper contamination.

In Werth (2006), different concentrations of several chemicals were tested in order to study early alarm induction in the DaphToxI®. In results, an alarm was triggered after 4h30 of exposure to 110 µg/L (>EC_{70} at 48 h) of **carbofuran**. The behavioral parameters that contributed to induce the alarm were the speed classes, the average speed and the height of swimming. The concentration of 0.0142 µg/L of carbofuran (EC_{20} at 48 h) tested in our study did not induce significant effects on the average speed. Thus, a high concentration of carbofuran seems to be needed to induce an effect in the DaphToxI® but the speed classes and the swimming height parameter should be investigated for carbofuran analysis. Furthermore, two other compounds sharing the same mode of action (AchE inhibitors), i.e., the organophosphate dimetoate and the carbamate carbaryl induced an alarm after 4h30 of exposure to a concentration well above the EC_{50} at 24 h. Again, behavioral response thresholds are high for these substances. For **cypermethrin**, the alarm threshold after 4h30 of exposure was equal to 2 µg/L (>EC_{70} at 48 h). The average speed and the swimming height mainly contributed to induce the alarm. The concentration of 0.09 µg/L (EC_{20}) of cypermethrin tested in our study did not induce significant results. Again, we can deduce that only concentrations in the range of the EC_{50} (48 h) can induce early alarms in the DaphToxI®. However, in her study (Werth 2006), Werth only focused on early alarm (4h30). We can thus hypothesize that concentrations between the EC_{20} and EC_{70} could potentially induce an alarm or a significant effect on the average speed over the 48 hours of exposure.

In conclusion, the concentrations needed to induce an alarm in the DaphToxI® are generally high (in the range of the EC_{50}). Similar pattern of increase of the average speed were observed in our results obtained from the DaphToxI® compared to the literature. This reinforces the hypothesis that behavioral effect onset can occur at different times according to the chemical used and that indirect toxic effects on behavior induced by chemical may be observed. Finally, in this study, we focused on the average speed in order to compare results with our new "Multi-DaphTrack" system. The average speed is not the only behavioral parameter that induces early warning signals. Other parameters such as speed classes, height of swimming, distance between organisms and the number of active organisms, could be monitored together with the "toxic index" in order to maximize the opportunity to detect chemicals with the DaphToxI®.

1.4 Relevance of the average speed as ecotoxicological endpoint

In our study in the "Multi-DaphTrack" system, the average speed was the most sensitive parameter compared to the number of active organisms and changes in path angle. In addition, for all tested compounds, the average speed increase was the earliest and most sensitive behavioral effect measured in the "Multi-DaphTrack" system. Whatever the considered chemical, the increased average speed was much more prominent over time than the decreased average speed. That is why our study on chemically induced behavioral effects was mainly **focused on the increase trends of average**

speed. The average speed parameter is by far the most frequently used parameter as early indicator of stress in *Daphnia magna* exposed to chemicals. For instance, the average speed has been found sensitive for sublethal exposures of organophosphates (Ren et al. 2009 (b)), cadmium (Baillieul and Blust 1999, Untersteiner et al. 2005, Wolf et al. 1998), cyanotoxins (Ferrao-Filho et al. 2014), nanoparticles (Artells et al. 2013, Noss et al. 2013). Our study showed that the average speed parameter is a suitable indicator of toxicity due to the low variability of the average speed between replicates and over time. Other behavioral parameters such as angular speed change of the number of active organisms may somehow be more difficult to exploit due to their high variability.

Nevertheless, other behavioral parameters may be also relevant and could be investigated to indicate chemical stress. For instance, **behavior strength parameter** (movement frequency) has been describes as a good indicator of early stress to acute pesticide exposure in *Daphnia magna* (accidental contamination) (Ren et al. 2007). Significant **changes in angle** and significant effect on **cumulative distance** have been reported for imidacloprid exposure at similar tested concentrations in *Daphnia pulex* (Zein et al. 2013). The measurement of **the phototactic behavior**, which is highly correlated with predation, would be a more representative bioindicator of field situation (Whitman and Miller 1982). Inhibition of phototactic responses have been, for example, observed in *Daphnia magna* exposed to naphthalene (Whitman and Miller 1982) or copper (Flickinger et al. 1982). In the latter study, copper concentration inducing negative phototactic (10 µg/L), was even lower than the behavioral LOEC found with the "Multi-DaphTrack" system. Trichlorfon or carbofuran, known as "nerve poison" could also impact the phototactic behavior and may alter daily the swimming activity (more visible for predators if they swim a lot). The measurement of **respiration** (consumption of O_2) may also be relevant since exposed organisms may try to remove the toxicant by increasing the flow over respiratory appendage (Stensberg et al. 2014). Another study also showed that the measurement of Chemical oxygen demand (consumption of O_2) in *Daphnia magna* could be a valuable tool for municipal sewage treatment plant monitoring (Tyagi et al. 2007).

In the DaphToxI®, many parameters are currently measured, i.e. the **number of active organisms**, **swimming speed distribution**, **fractal dimension** (turning rates and circling movement, curviness)**, swimming height and distance between organisms**. Under normal conditions of DaphToxI® use, these parameters are usually integrated in a toxic index. These parameters contributed in various degree levels to induce alarms under chemical exposures (Chancerelle et al. 2010, Lewandowska 2004, Werth 2006). The average speed class distribution, which is a complementary parameter of the average speed, could be also considered as an indicator of toxicity. For instance, when toxicity occurs, daphnids may swim in different average speed class.

Other behavioral parameters have been investigated in invertebrates. For instance, the following of **swimming state frequencies** with break, cruising and sinking has been measured on the

calanoid copepod *Eurytemora affinis*. This parameter was shown to be sensitive to sub-lethal concentrations of nonylphenols, cadmium and mixture of polycyclic aromatic hydrocarbons (Cailleaud et al. 2011, Michalec et al. 2013). This parameter could be also applied to *Daphnia magna* by differentiating the distribution of swimming speed and measure the frequencies of period of "break", slow and fast swimming. The **swimming angular** and **linear speed alteration** have been found sensitive in the rotifer *Brachionus calyciflorus* exposed to sub-lethal concentrations of dimetoate (Guo et al. 2012). **Moving velocity and moving distanc**e were utilized in the marine shrimp *Hippolyte inermis* (crustacean decapod) to detect sub-acute effects of cadmium (Untersteiner et al. 2005).

2. ANALYSIS OF DAPHNIA BEHAVIORAL EFFECTS AND ITS UTILITY IN RISK ASSESSMENT

2.1 Understanding of behavioral effects according to the tested chemical

Profile of the average speed increase was different between tested compounds in the **time of response**, the **duration** and the **intensity** of effects. In this study, the effect onset of toxicants has been observed to be early or delayed according to their mode of action or the mechanism of action and hence highlights the **importance of following effects over time**, which may help in the determination of compounds with transient or persistent behavioral effects. For instance, exposure to narcotics induced an intense and rapid increase of the average speed followed then by a gradual decrease of the average speed while the effects of specific or multiple modes of action compounds were more heterogeneous and characterized either by a steep or by a slight increase followed by a rapid slowdown back to or under the control level. The time of effect onset also differed between specifically acting compounds with delayed, intermediary or rapid onset of effects.

2.1.1 Do compounds sharing the same mode of action show similar behavioral effects?

Our starting hypothesis was to determine if behavioral profiles could be distinguished based on the chemical mode of action. As it is explained in the **chapter III,** no distinct behavioral profiles could be drawn from the chemical mode of action. Thus, the interpretation of effect profiles may not always be straightforward and specific interactions with multiple target sites have to be considered.

Both **isopropanol** and **ethanol**, acting through narcosis, showed similar time course effects on the average speed (i.e., an intense increase followed by a gradual decrease). For lipophilic narcotics, uptake of the compound is governed by partitioning into biological membranes (Escher and Hermens 2002). No active transport process or metabolism is involved, which can explain the immediate behavioral response that was observed (time for daphnia to reach equilibrium with the surrounding aqueous phase). The "narcotic" compounds surprisingly induced long-lasting behavioral excitations at low concentration exposure while "narcosis" is commonly related to reversible anesthetic effects, i.e., depressed locomotion (hypoactivity syndrome), as noted by Drummond and Russom (1990).

Contrasted profiles of the average speed were observed for specifically acting chemicals. The intrinsic toxicity of specifically acting compounds may be dependent on their affinity to, or type of interaction with, the target receptor or the internal chemical speciation (Escher and Hermens 2002). **Esfenvalerate** and **cypermethrin** share a similar mode of action as a modulator of sodium channels, and both exhibited a rapid effect on the average speed. Although **fipronil** and **abamectin** have dissimilar modes of action (opposite mechanisms on GABA receptors, i.e., antagonist and agonist, respectively), both induced similar rapid behavioral effects. However, among compounds belonging to the **same mode of action class** (e.g., neurotoxics), **differences** in effect intensity, time to effect onset and duration of effects may be observed. **Carbofuran** exposure showed slight effects on the average speed and at the end of the exposure, the average speed went back to the control level. This pattern reflected a rapid recovery of the AchE enzyme. **Trichlorfon** exposure induced more significant but delayed effects on the average speed. These differences might be explained by difference in the toxicokinetic behavior of the compounds or subtle differences in the mechanism of action, i.e., phosphorylation and carbamylation of the AchE enzyme by trichlorfon and carbofuran, respectively, or carboxylesterase inhibition (Barata et al. 2004). Another piece of evidence against our hypothesis is that chemicals with dissimilar modes of action can show similar behavioral effects, e.g., **isopropanol** and **esfenvalerate** have **similar effect profiles** but **dissimilar modes of action.** A recent study dealing with the behavioral responses of copepods exposed to nonylphenol, cadmium, and a PAH mixture, showed that narcotic and non-narcotic pollutants induced both hyperactivity. As our results, this study suggests that the behavior after a short-term exposure was not dependent of the general mode of action (Michalec 2013). $EC_{50,exp}$ (48 h) values for specifically acting compounds with high hydrophobicity (i.e., $\log K_{ow}>5$), e.g., cypermethrin, and esfenvalerate, tend to deviate less from baseline toxicity than hydrophilic compounds. Despite that sertraline and fipronil are well recognized as specifically acting compounds, their respective excess toxicity is between $-1<\log T_e<1$, indicating baseline toxicity. In this case, the "dominant" mode of action for this compound may be narcosis instead of neurotoxicity. When the log K_{ow} is high, the toxicity of specifically acting compounds may be mainly driven by hydrophobicity because of a high adsorption of the compound on the cuticle and in the cellular membranes. Our results emphasize the fact that interpretations of behavioral profiles may not always be straightforward, and specific interactions with target sites must be considered.

2.1.2 The cause of behavioral responses: sensorial perception or toxic action of the chemical?

According to Charoy et al. (1995), behavioral endpoints can be considered as an integration of physiological, metabolic, sensorial, nervous and muscular systems. Behavioral effects may hence be due to maintenance cost to restore imbalance, to chemical detoxification and excretion processes, to toxic action on the central nervous system or/and exhaustion of the exposed organism. Under toxic exposure, organisms can change their behavior (e.g., ventilation rate, locomotion) and flee the

contaminated area to avoid or limit toxicant exposure. Chemical detection can be done by visual or chemoreception (olfaction, taste) or after cutaneous irritation. Based on previous assumptions and exposure data, we can describe processes of the stress response of *Daphnia magna* to a chemical exposure.

Presumably, the increase of the average speed observed just after the onset of chemical exposure may characterize avoidance, i.e. a stress response of the organism trying to escape the contaminated area. The avoidance response may reduce exposure to harmful conditions and avoidance failure may lead to reduced fitness, or worst to deleterious effects. Avoidance in response to toxicant stress is well documented in fish and crustacean (Atchinson et al. 1987, De Lange et al. 2006, Eriksson Wiklund et al. 2006, Hellou et al. 2005, Kravitz et al. 1999). For *Daphnia magna*, avoidance responses to olfactive cues from conspecifics and predators have been highlighted (Dawidowicz and Loose 1992, Roozen and Lürling 2001, Stirling and Roff 2000). Besides, evidences shown that Daphnia are able to detect the presence of kairomones released by predators (Hazanato 1999). Therefore, we may think that daphnia possess chemoreception (olfaction or taste) for chemicals and exhibit avoidance behavior under toxicant stress. The increase of the average speed is by far the most frequently reported behavioral effect and is commonly used as an early signal of stress in *Daphnia magna* exposed to various chemicals. For instance, avoidance behavior of *Daphnia magna* was used as an indicator of early stress following an accidental organophosphorus pesticide (OP) contamination (Ren et al. 2007, Ren et al. 2009 (b)). In our study, the average speed was the most sensitive parameter compared to the two other parameters investigated, i.e., the number of active organisms and changes in path angle. Furthermore, the average speed increase, which may indicate avoidance, was earlier and much more contrasted than average speed decrease, and hence appears as a suitable stress indicator. No direct link can be established between mode of action and behavioral effects. Daphnia possess olfactory senses allowing to detect cues from food, competitors and predators (Roozen and Lürling 2001). One hypothesis is that daphnia are able to sense chemical cues. However, the ability to sense chemical cues is inhibited by certain contaminants, and behavioral changes may in this case, directly be correlated to toxic impacts (Lürling and Scheffer 2007).

On the other hand, the **increase** of the average speed at latter time of exposure may not be an avoidance response but rather a consequence of adverse physiological effects induced by toxicant exposure (i.e., alarm reaction due to effects). Hence, another possible explanation is when animals are suffering, **indirect effects** may occur in behavior. Invertebrates are generally assumed to be insentient, or at least less sensitive than vertebrates. Notwithstanding, a few researchers have investigated if invertebrates are able to experience pain and this controversial issue has been discussed for crustacean decapods (Gherardi 2009). It was shown that disturbance of behavior may also come from pain induced by the toxic. We can postulate that daphnia are likely **able to experience pain** and may consequently exhibit **unconscious reflex behavioral responses** to pain or tissue-damaging stimuli. A

recent study also demonstrated that the crustacean crayfish exhibit a form of anxiety similar to that described in vertebrates suggesting the conservation of several underlying mechanisms during evolution (Fossat et al. 2014). The Figure 25 illustrates the possible causes in the different observed behavioral effects.

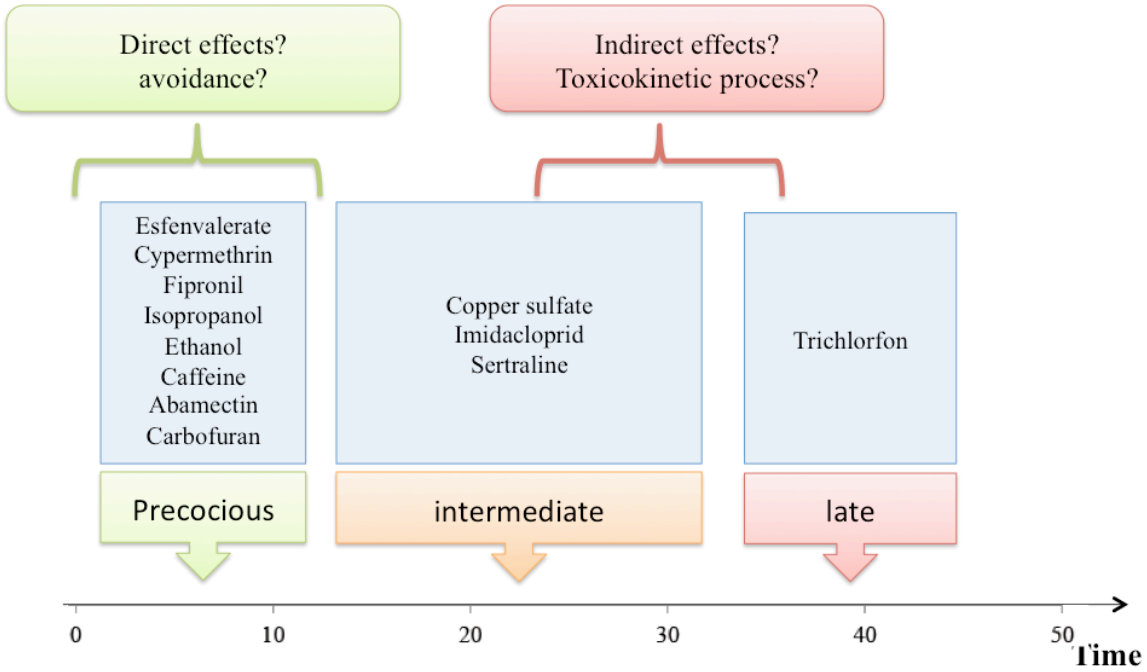


Figure 25: The cause of behavioral responses: sensorial perception or direct/indirect toxic action of the chemical.

The **decrease** of the average speed may induce a depressed muscular activity, which may be due to a loss of coordination or the higher maintenance costs for protection mechanisms. Indeed, energy resources of the organism are limited; the global metabolic rate in *Daphnia magna* does not increase under exposure. Thus, the additional metabolic costs to cope with toxicant exposure necessarily imply a reallocation of energy resources in non-muscular tissues (Knops et al. 2001).

2.1.3 Evolution of effect over time

Both isopropanol and ethanol, acting through narcosis, showed similar time course impact on average speed (i.e., intense increase followed by gradual decrease). Ethanol results showed stimulatory effects (i.e., increase of the average speed) at low concentrations. For the highest concentrations, stimulatory were less intense and tend more rapidly to decrease toward inhibitory effects (i.e. decrease of the average speed). Ethanol affected behavior in a non-linear manner suggesting already reported nonlinear effects of ethanol on the behavior of zebrafish (*Danio rerio*) (Gerlai et al. 2000). Ethanol was shown to have an inhibitory effect at high doses and a stimulation effect at low doses on locomotion (i.e., locomotion activity). These results highlight that behavioral effects may follow a

biphasic dose-response curve rather than linear. For metal exposure, a wide range of physiological toxic processes are induced including neurological effects, metallothionein synthesis induction etc. For instance, important effects on the nervous system may occur and may impact behavioral responses. Metallothioneins are proteins, which bend to metal cation to reduce toxicity of metals. As it is assumed by Untersteiner et al., (2005), these proteins, which play a major role in detoxification process of metals, may be induced during the phase of non-observed effects (i.e., the first 5 hours of non-observed behavioral effects for copper exposure to concentration below the EC_{50} (48 h)).

Toxicokinetic processes are likely involved in the time of effect onset, since the toxicant needs to go through the epidermal tissue to reach the target site (e.g. nervous system for neurotoxics) and to exert its adverse effects. **Immediate responses** were observed for half of the specifically acting chemicals tested, i.e. **esfenvalerate, fipronil, cypermethrin, abamectin, caffeine and carbofuran** and may result from a fast absorption and tissue distribution of the compounds. **Copper sulfate** exposure induced **intermediary significant increase** of mean speed ($p < 0.01$) from 5 to 11 hours for all tested concentrations. This observed delay for behavioral effects may be explained by the time needed for metal uptake, which depends on speciation of copper. The relatively fast recovery of Daphnia exposed to copper sulfate may be related to protective mechanisms such as intracellular metal sequestration through metal-binding proteins (i.e., metallothioneins) or detoxification mechanisms (e.g., elimination through the sodium, potassium or calcium channels of the cellular membranes mediated by specific transport systems such as cation ATPases, esterases, monooxygenase cytochrome P-450, MFOs, gluthation-S-transferases and hydrolase epoxy). For instance, it is known that fish degrades more slowly **cypermethrin** compared to other mammal species, and enhance the toxicity of cypermethrin to fish (Bradbury and Coats 1989). On the other hand, chemical are often metabolized in more toxic metabolites and increase the toxicity of the chemical exposure. When a significant decrease of the average speed was observed at the highest concentration (>EC_{70} (48 h)), protective mechanisms are likely to be saturated and then deleterious effects can occur. These results are in accordance with a previous study (Jeon et al. 2008), which reported hyperactivity at 7 hours for a concentration of 50 µg/L of copper sulfate. **Imidacloprid** also showed an **intermediate delay** in the behavioral responses. Surprisingly, a previous study reported rapid increase of cumulative distance swum (which is proportional to average speed) by *Daphnia pulex* exposed at concentrations of 65 mg/L of imidacloprid (Zein et al. 2013). Behavioral effects induced by **sertraline** exposure were also **intermediate**. This delay of responses may indicate the time needed for the chemical to be taken up and to exert toxicity in the organism. An efficient defense mechanism may be also involved in the delay of response, preventing chemical effects to occur until these defenses get saturated. Unfortunately, sertraline is an emerging substance and data is still too scarce to support this hypothesis. **Trichlorfon** exposure produced unexpected results: behavioral effects were very delayed and occurred at concentrations similar to the time of immobilization effects. However, this delay of

behavioral responses was already observed in Daphnids exposed to trichlorfon in the DaphToxI® (Lewandowska 2004).

Behavioral effect pattern over time are described in the literature with several models. For instance, regression-based model method has been developed by incorporating hormesis for the determination of EC_{50} (Vanewijk and Hoekstra 1993). However, the use of hormesis model in risk assessment is not widely accepted (Chapman 2002). If hormesis occurs in behavioral assessment, behavioral NOEC determination has then low utility for risk assessment (Chapman 2002, Forbes 2000). Hormesis is basically simply a response to stress, which can induce positive (adaptive), neutral or adverse (maladaptive) effects and can be explained as an evolutionary adaptation that acts to maintain fitness in a changing environment. The utility of NOEC is in question in regulatory process and may be not beneficial to ecosystem (for instance for essential metals). Gerhardt et al. have also described a behavioral pattern after toxicant exposure over 48 h-exposure time with a Stepwise Stress Model SSM (Gerhardt et al. 2005). This model highlights the sequence of behavioral stress responses above threshold of resistance to chemical exposure. According to this model, the decrease of the average speed may be related to different things including acclimatization to the stress or toxicity. For instance, this model has been taken back by Ren et al. (2009 (b)) for consequential reactions over 48 hours under organophosphorus pesticide stress. The Figure 26 described the behavioral pattern over time including 6 different phases: 1) no effect, 2) stimulation, 3) acclimation, 4) adjustment, 5) toxic effects and 6) readjustment.

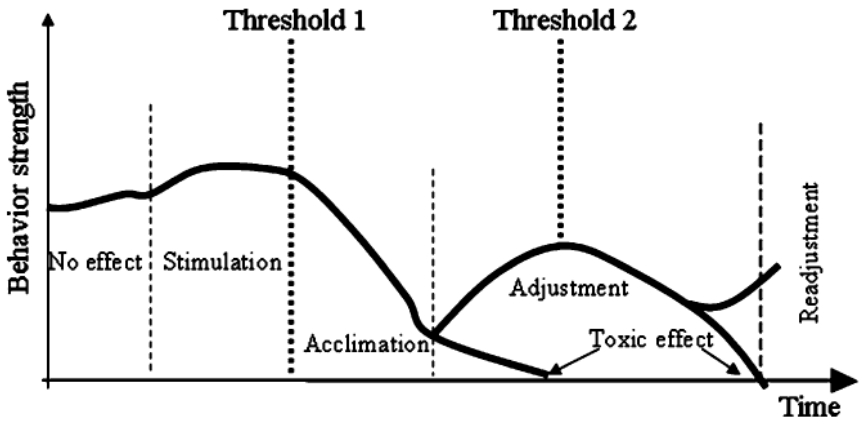


Figure 26: The theoretical reactions of *Daphnia magna* in a certain behavior under OP stress over 48 hours (Ren et al. 2007).

2.2 Comparison with other toxicity tests performed on *Daphnia magna*

Various tests are currently performed to assess effects on *Daphnia magna* induced by chemical stress (e.g. mortality, morbidity, growth, reproduction, heartbeat, behavior, biochemistry, respiration, cytogenetic, cytology, embryology, carcinogenesis, etc.). The chronic toxicity test, which usually uses reproduction or growth parameters assessment over 21 days, is often the most sensitive

test. Behavioral results obtained in the "Multi-DaphTrack" system can be compared with chronic toxicity data (reproduction test, 21 days) available in literature and on-line databases (see Table 6).

Table 6: *Daphnia magna* chronic toxicity data from literature and on-line database compared to the behavioral LOECs obtained from the "Multi-DaphTrack" system. (a: (USEPA 2014), b: (Jemec et al. 2007), c: (Minagh et al. 2009), d: (Muyssen and Janssen 2007), e: (Firpo 2011), f: (Matsumoto 2009)).

Substance	Behavioral test	Chronic test	
	LOECs (mg/L)	NOECs (mg/L)	LOECs (mg/L)
Isopropanol	<1300	N.D.	N.D.
Ethanol	4800	**16**[a]	**0.01**[a]
Caffeine	25	N.D.	N.D.
Imidacloprid	85	**1.5**[b]	**2.5**[b]
Sertraline	0.03	**0.032**[c]	**0.1**[c]
Copper sulfate	**<0.028**	**0.075**[d]	**0.09**[a]
Fipronil	**<0.001**	**0.010**[a]	**0.019**[a]
Carbofuran	**<0.011**	**0.01**[a]	**0.027**[a]
Esfenvalerate	**<0.0001**	**0.00011**[e]	N.D.
Cypermethrin	0.00004	**0.0000009**[a]	**0.000002**[a]
Abamectin	**<0.00004**	**0.00003**[a]	**0.00009**[a]
Trichlorfon	0.00034	**0.000006**[a]	**0.001**[f]

As behavioral NOEC determination was not possible for most of tested compounds, only behavioral LOEC are compared to chronic toxicity data. Overall, the behavioral assay is globally less sensitive than the chronic assay for ethanol, imidacloprid and cypermethrin (behavioral LOECs above the NOECs and LOECs of the Chronic tests). The behavioral LOEC of trichlorfon is higher than the NOEC but lower than the LOEC of the chronic toxicity test. For **sertraline, carbofuran, esfenvalerate and abamectin,** our behavioral results showed **similar toxicity thresholds** compared to **chronic toxicity data (NOECs)** and for **copper sulfate pentahydrate,** the **behavioral responses were more sensitive** than the chronic toxicity endpoints. No chronic data is available for isopropanol and caffeine. Altogether, the chronic toxicity assay tends to be more sensitive than the behavioral assay. The higher sensitivity of chronic tests is not surprising, this is obviously due to a longer exposure time over one generation cycle in comparison to 48h exposure for our behavioral assay. Nevertheless, the time needed to perform a chronic test is much longer than the behavioral test (21 days vs. 48 hours). Hence, we can conclude that the behavioral test still represent great advantages compared to the standard chronic test since **behavioral change thresholds are not so far from chronic toxicity thresholds** and **behavioral tests** are **easy** to perform and **not time consuming**. It is noteworthy that the determination of behavioral NOECs was not possible for most of tested

compounds because significant effects were observed from the first tested concentrations. In future experiments, lower concentrations should be tested for the determination of NOECs.

2.3 Ecological relevance of behavioral endpoints analysis for environmental risk assessment

Our results show that chemical exposure can lead to significant alterations of the swimming parameters in few hours after the beginning of the exposure and at low concentrations, which is in line with experimental results from the literature. These early responses could allow rapid detection of toxicants in water. Behavioral tests are ecologically relevant and have therefore become of interest in water quality assessment as an alternative to, or supplement to chemical monitoring. Behavioral tests are more sensitive than the acute standard test currently used to assess the toxicity of substances released in the environment. When evaluated by using acute standard test, the chemical risk may be underestimated. In this case, behavioral test, which is more sensitive, could be used to evaluate the chemical risk and hence enhances the risk assessment reliability. The comparison of the behavioral thresholds of tested chemical to chronic toxicity data found in literature indicates that the chronic toxicity assay tends to be more sensitive than the behavioral assay. However, behavioral thresholds are quite closed to chronic toxicity data for some of the tested compounds and may potentially be considered as indicator of chronic toxicity. Previous studies have already discussed about the consideration of behavioral changes as indicator of chronic toxicity in *Daphnia magna* (Flickinger et al. 1982, Whitman and Miller 1982) or in amphipod (Scarlett et al. , Ward et al. 2013). These studies proved that sublethal concentrations can critically effect the behavioral responses of organisms. To date, behavioral assays are not taken into account in regulatory guideline TGD for the evaluation the risk of chemicals (2000/60/EC, 2011). However, no single biological technique can determine "safe limits" for water quality control and it should be preferred to use all available methods to assess pollutant effects (Whitman and Miller 1982). As reported by the American Society for Testing Material (ASTM) in the standard guide for behavioral testing in aquatic organisms (ASTM 2012), behavioral toxicity test methods may be used to predict the effects of long-term exposure and be useful for long-term monitoring of effluents. Behavioral thresholds may be considered as suitable indicator to prevent chronic toxicity and might be taken into consideration for assessing the hazard of chemical to aquatic organisms. In this way, **the behavioral NOEC and LOEC** could be used for the **PNEC calculation** in risk assessment and may be useful for guiding decisions regarding the extent of remedial action needed for contaminated aquatic and terrestrial sites. Furthermore, the ASTM report also indicates that additional behavioral methods for any category may be added when new tests are developed as well as when methods are adapted to different species or different life stages of an organism (ASTM 2012). Hence, we think that our new daphnia behavioral system could be added in this standard behavioral guide.

The other main point to evaluate the relevance of behavioral endpoints is to known if a significant behavioral effect results necessarily in a toxic effect. Furthermore, it is not clear if behavioral effects themselves could lead to ecological impacts and could be considered as toxicity or not. In an ecological context, behavioral responses to low levels of pollutants may be interpreted as **positive** (e.g. adaptive) or **negative** (e.g. toxic) responses. Behavioral responses, if reversible and under a certain threshold intensity, may not induce adverse effects or impact survival. On the other hand, significant alterations of the mobility can also impact the fitness and the survival of organisms, which may lead to long-term changes at population and community levels (Duquesne and Küster 2010). Indeed, if swimming speed increases, the energy used for normal metabolic functions (e.g. growth, reproduction and locomotion) can be reallocated to locomotion and may therefore impact long-term survival (Knops et al. 2001). Sub-lethal effects typically occur at concentrations below those producing direct somatic death but this concept of sub-lethal effects is somewhat ambiguous as they can still have lethal consequences. Indeed, when considering indirect effects in a real ecological context, some sub-lethal effects (including behavioral effects) can lead to lethal consequences since daphnia survival is dependent on competition with conspecifics and other species, predation avoidance, search for food and other multiple stressors (Newman and Unger 2003). For instance, adaptive behavior such as avoidance or alteration of mobility under toxic stress can impact decision-making (e.g. location), and organisms may be more noticeable to predators or less able to avoid predators and thus potentially increasing the predation rate (Reynaldi et al. 2011). On the other hand, behavioral effects such as escape may be considered as a beneficial response: the increase of the swimming speed allows the exposed organisms to avoid the contaminated area or predation. Avoidance has been already reported in the literature for crustacean (De Lange et al. 2006, Eriksson Wiklund et al. 2006, Hellou 2011, Kravitz et al. 1999). A decrease of the swimming speed may also reduce energy cost, which is reallocated for detoxification mechanisms, and make the organisms less noticeable for predators. On the contrary, an increase of the swimming speed leading to an overconsumption of energy may reduce energy allocation for less vital functions such reproduction, growth etc. As “beneficial effects” on one endpoint (i.e. the swimming speed), may not be beneficial for the overall fitness of the organism (Forbes 2000). Besides, we must keep in mind that even positive effects from chemical exposure may negatively impair another species and then disturb the ecological balance of ecosystems. As a keystone species, effects on *Daphnia magna* may lead to indirect effects on other organisms by impacting, for instance, the lower levels as a prey or the higher levels as a predator. Individual survival is not of concern for ecological risk assessment but rather protection of the whole ecosystem is important to preserve biodiversity.

2.4 The use of behavioural thresholds for risk assessment of the studied pollutants

2.4.1 Chemical concentrations occurring in the environment

A recent large-scale study encompassing 4000 European monitoring sites, revealed that nearly half of European water bodies are subjected to organic chemical pollution threatening ecological integrity and loss of biodiversity (Malaj et al. 2014). This study reported that 14% of the monitoring sites were likely to be acutely affected by the organic chemicals and 42% were likely to be chronically affected by organic chemicals for at least one organism group (Figure 27). For invertebrates (i.e., Daphnia data), maximal concentrations of organic chemicals are above the acute risk threshold at 6% of measured sites, and the mean chemical concentrations exceed the chronic risk threshold at 38% of sites. This study is the first risk assessment on the continental scale and shows that there is a global concern (not only localized) comprising both aquatic ecosystem and human health. The immobilization measured as effect parameter in the standard protocol at two pre-defined exposure time of 24 hours (usually for effluent assessment) and 48 hours (for other substances) is not sensitive enough to account for sub-lethal effects that can be induced by low concentrations that typically occur in surface waters. Fortunately, concentrations inducing mortality are rarely reached in the aquatic environment. With behavioral systems, we may observe effects that cannot be detected by chemical analysis. Furthermore, behavioral tests may complete information collected by standard toxicity tests and provide more ecological relevant data.

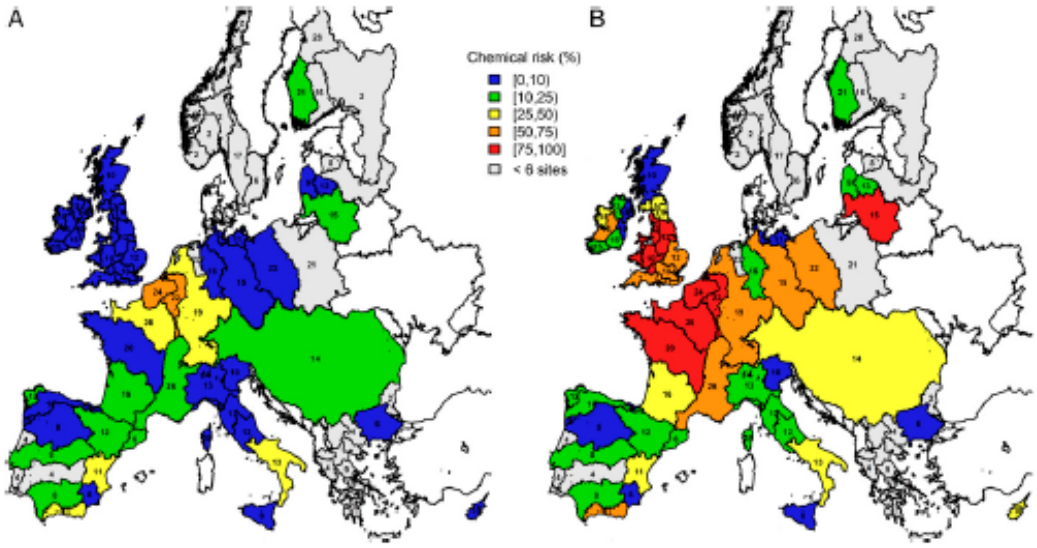


Figure 27: Chemical risk (by % range) in European river basins. The map (A) depicted concentration exceeding the acute risk threshold and (B) exceed the chronic risk threshold (Malaj et al. 2014).

Of the 223 chemicals included in the European surface water monitoring (Malaj et al. 2014), AchE inhibitors including carbamates and OPs and sodium channel inhibitors (pyrethroids) were major contributors to the chemical risk. Unfortunately, of our tested substances only carbofuran was included in the monitoring: concentrations ranging from 0.015 and 5 µg/L were measured on the European continent (see Table 7). Maximal concentrations are only slightly below the behavioral LOEC (11 µg/L) obtained in the "Multi-DaphTrack" system. Measured environmental concentrations for the other tested substance can be found in literature with punctual and localized measurements (see Table 7). For instance, a concentration of 856 ng/L of caffeine found in French river has been reported by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) during a national survey of 9 months comprising 238 sites all over France (Bouissou-Schurtz et al. 2014). Hence, the toxicity assessment for caffeine may not be of priority since toxicity thresholds are far above the measured environmental concentrations. The highest concentration found in Swedish surface waters for esfenvalerate exceeded chronic toxicity data (NOEC of 0.11 µg/L) and was higher than the Water Quality Standard value (WQS equal to 0.0001 µg/L) (Firpo 2011). The "Multi-DaphTrack" system could be able to detect effects at such environmental esfenvalerate concentration since the behavioral LOEC obtained with our "Multi-DaphTrack" system (0.1 µg/L) was lower than the MEC. The DaphTox® may also be able to detect this environmental concentration since an alarm has already been obtained at 0.1 µg/L (Lewandowska 2004). However, by only measuring the average speed change, the DaphTox® could not be able to detect this measured environmental concentration since significant changes in the average speed were observed only from 0.035 µg/L (Chevalier et al. 2014). Imidacloprid, which is the most-used insecticide in Netherlands or California is persistent in water, not easily biodegradable and is likely to remain in the water column (Van Dijk et al. 2013). Imidacloprid is the most-used insecticide in Netherlands and California. The highest concentration measured in the environment in the Netherlands is equal to 320 µg/L, which is below our behavioral threshold. Sertraline is a highly consumed pharmaceutical product that is emitted in high amount from e.g. hospitals. Sewage treatment plants do not eliminate sertraline and the measured concentrations in released treated effluent of water treatment plants in Norway range between 0.017 and 0.0024 µg/L (Ahlford 2004). The regulation level of trichlorfon for drinking water in Japan is 30 µg/L. With the "Multi-DaphTrack" system, we can detect concentrations as low as 0.34 µg/L.

Concentrations of the tested substances inducing an alarm in the DaphTox® or significant effects on the average speed in the "Multi-DaphTrack" system do not currently occur in the environment. However, when considering accidental concentrations reported in the literature, these thresholds could be exceeded in case of accidental spilling. For instance, the accidental discharges in the Breitenbach stream in 1986 has lead to a concentration of 91 µg/L of cypermethrin in the stream inducing the complete intoxication of arthropods present in the stream (Zwick 1992). This high pollution of cypermethrin could be detected, for example, with the DaphTox®, since a concentration

of 1 µg/L has been reported to induce an alarm (Werth 2006) or with the "Multi-DaphTrack" system (LOEC of 0.04 µg/L). **A maximal concentration of 26 µg/L of carbofuran** was also already measured in streams nearby agriculture activities (Matthiessen et al. 1995). This pollution by carbofuran could be detected with the "Multi-DaphTrack" system (LOEC of 11 µg/L).

2.4.2 Risk assessment for studied pollutants

For most substances, the pool of toxicity data can be limited. When only short-term toxicity data are available, the assessment factor used is empirical and high (1000) and it is well recognized that it does not provide a strong scientific validity. It is more a likelihood that an unacceptable effect will not occur than ensuring an overall protection of the environment. If behavioral responses may be considered as chronic toxicity, behavioral thresholds such as LOEC or NOEC could be used in risk assessment and contribute to the calculation of PNEC threshold. PNEC values available in the literature are listed in the

Table 7.

Table 7: Behavioral thresholds from the "Multi-DaphTrack" system compared to measured environmental concentrations and predicted no effects concentrations collected from the literature. a: (Bouissou-Schurtz et al. 2014), b: (Lamichhane et al. 2014), c:(Besse 2010), d:(OECD SIDS 2003), e:(Vasskog et al. 2006), f: (database 2014), g:(Van Dijk et al. 2013), h: (Firpo 2011).

Substance	Behavioral LOECs (µg/L)	MEC (µg/L)	LOQ (µg/L)	PNEC	
				Value (µg/L)	α
Caffeine	25000	0.856[a]	0.25	87[f]	1000
Imidacloprid	85000	320[g]	-	0.6[f]	1
Sertraline	30	0.06-6[b]	-	0.090[c]	100
Copper sulfate	<28	0.0005-104800[f]	0.0005	1.6[f]	2
Fipronil	<1	0.0005-0.0348[f]	-	0.00077[f]	10
Carbofuran	<11	0.015-5[f]	0.015	0.02[f]	10
Esfenvalerate	<0.1	0.2[h]	-	0.08[f]	2
Cypermethrin	0.04	<LOQ[f]	0.005	0.01[f]	2
Abamectin	<0.04	-	-	0.0163[f]	2
Trichlorfon	0.34	0.03-182[e]	-	0.00056[f]	10

For instance, a PNEC value was calculated for **caffeine** in (OECD SIDS 2003) following the EU risk assessment procedure (TGD, 2011) and was equal to 87 µg/L by applying an assessment factor of

1000 on the most sensitive species *Leuciscus idus* (LC_{50} 96h of 87 mg/l). No chronic toxicity data are available in the literature. Based on the behavioral NOEC of 12.5 mg/L, we can calculate a new PNEC of 125 µg/L by applying an assessment factor of 100 on the value of NOEC. With this new estimated PNEC, the risk of caffeine on the entire ecosystem is still negligible, since environmental concentrations are usually less than 1 µg/L. A PNEC of 0.77 ng/L has been established for **fipronil** based on a NOEC of 7.7 ng/L using a safety factor of 10. Interestingly, our behavioral LOEC is inferior to this value (1 ng/L). By considering this behavioral LOEC, the PNEC value could be estimated to 0.1 ng/L by applying a safety factor of 10. The PNEC of **imidacloprid** is equal to 0.6 µg/L and is based on a NOEC value of 0.6 µg/L from a mesocosm study with a safety factor of 1. The behavioral thresholds found in the "Multi-DaphTrack" system for imidacloprid (behavioral NOEC and LOEC are equal respectively to 34 and 85 mg/L) are much higher and do not represent much interest for PNEC evaluation. PNEC values for **esfenvalerate, cypermethrin and abamectin** are determined based on mesocosms tests and are well representative of the ecosystem. The safety factor is consequently very low and the behavioral NOEC or LOEC are higher than those obtained from mesocosm experiments. The safety factor for the calculation of **the copper sulfate** PNEC is very low as well. For these substances, behavioral tests do not provide particular relevant information. However, it is noteworthy that the behavioral NOEC and LOEC are in the same range compared to values of chronic toxicity. For **carbofuran**, the PNEC was calculated based on a NOEC of 0.2 µg/L, which is lower than our behavioral LOEC of 11 µg/L. The assessment factor (equal to 10) cannot be decreased with an additional behavioral NOEC/LOEC (3 NOECs from different trophic levels are already available).

Risk management has to deal with the balance between aquatic ecosystem protection and economic stakes. Overestimated or underestimated effects are not wished. **When chronic toxicity information is missing, behavioral tests may complete information obtained from standard toxicity tests and provide more ecological relevant data for the calculation of PNEC.** Furthermore, behavioral thresholds may be also more sensitive than other chronic or acute toxicity data for some chemicals. Linking the adverse effects of these impacts in individual animals to their ecosystem-level consequences is a key challenge in regulatory risk assessment (Moore et al. 2004). PNEC extrapolation is of course heavily influenced by the species-specific toxicokinetics and toxicodynamics of chemical stressors. Uptake is influenced by surface to volume ratio, so effects may be less important on adult daphnia and other larger species. The extrapolation of the results provided by single taxa to ecosystem undoubtedly involves a number of uncertainties. Ecological risk assessment is rarely focused on individual organisms but rather on populations, communities and ecosystems (with interactions with their abiotic surroundings), which are rarely disturbed by losses of individuals. The most important is the stability of a particular population size and structure, and to prevent irreversible reductions that could lead to extinction. The risk assessment should protect species composition because it ensures the protection of ecosystem functions. The use of single taxa impairs

extrapolation to the state of an entire system since complex interactions can occur in ecological network (Goodsell et al. 2009, Gray et al. 2014). Despite *Daphnia magna* is sensitive to an extended range of chemicals; behavioral studies should be also performed on other species from different trophic levels. For toxicity assessment of complex mixtures of pollutants, complementary behavioral studies on various species from different trophic levels may obviously add values to risk assessment. Furthermore, experiments are conducted on daphnia clones to reduce biological variability, which do not represent the natural variability of wild populations. Hence, results are likely not representative of the natural plasticity in behavior in field condition (Boersma and Spaak 1998).

3. BEHAVIORAL ANALYSIS SYSTEMS IN RISK ASSESSMENT

3.1 Evaluation of biomonitor utility for biomonitoring programs

As an example of BEWSs, we have exposed Daphnids to different chemicals in the DaphToxI®. This system, as the other BEWSs, can be used ***in situ***. So far, continuous measurement providing responses over time appears as a major advantage of on-line biological early warning system when aiming an early detection of pollution waves or short-term pollution peaks, which might be miss out by classical spot sampling and chemical target analysis. Furthermore, laboratory experiments do not take in account the full set of naturally occurring abiotic and biotic variables, which can influence the contaminant bioavailability, altering the uptake of the contaminant by the organisms and which then may change the response of organisms (Goodsell et al. 2009). By exposing organisms to *in situ water*, BEWSs conditions of exposure get closer to real field conditions (i.e., pH, hardness, etc. Another important point is that environmental pollutant concentrations may be under the limit of quantification of chemical analysis method and BEWS may detect environmental pollutant concentration, which cannot be detected with physico-chemical measures (Suter and Glenn 2001).

Similar trends of swimming speed increase were observed for some chemicals (especially for esfenvalerate) in the DaphToxI® compared to the "Multi-DaphTrack" system. Some discrepancies in behavioral responses were observed for some chemicals in regard to "Multi-DaphTrack" system (e.g. imidacloprid). Exposure at a concentration of 85 mg/L of imidacloprid induced a significant decrease of the swimming speed in the DaphToxI® system whereas significant increase of the swimming speed was observed in the "Multi-DaphTrack" system. Imidacloprid is known to induce disorientation in Honey Bees (Bortolotti et al., 2003). In contrast to static conditions, flow-through conditions may enhance the disorientation of exposed Daphnids. Overall, direct comparison should be considered with caution since these two systems have different exposure conditions (i.e. static/flow-through) and that DaphToxI® system can only perform one replicate and does not allow statistical analysis. Furthermore, we must keep in mind that we did not utilize the DaphToxI® in normal conditions of utilization. Indeed, the DaphToxI® is initially designed to observe various behavioral parameters, which are integrated in a toxic index. When the toxic index exceeds a predefined threshold, an alarm is triggered. Hence, other parameters than the swimming speed should be also investigated in the DaphToxI®

instead of focusing on only one parameter. The use of the toxic index already provided interesting results and allowed the identification of pollution events in surface waters (De Hoogh et al. 2006). Obviously, the water quality monitoring of all small streams and rivers is not possible, because of the high cost it would imply. However, punctual controls or control when a possible pollution is suspected should be conceivable. For this to be achieved, mobile structure should be used in priority, such as the **mobile station** built by EDF and INERIS.

The DaphToxI® is originally designed to detect a significant deviation from the previous recorded-behavior. The concept of analysis with the DaphToxI® is quite different from the usual standard tests performed in ecotoxicology, which mainly compared chemical induced effects of a direct exposure to a group of controls. This is due to the fact that for surface water monitoring, control water (i.e., water sharing the same physic-chemical conditions but without chemicals) is not available. Therefore, the comparison of measurement with the preceding behavior (e.g., mean of preceding 10 to 30 minutes) is a pragmatic choice for detection of deviation of normal behavior. In its normal functioning, the daphnids are kept until seven day (before the first brood) and are fed with an algae solution. Therefore, several factors that may influence results should be considered seriously. For instance, as it was observed by Chancerelle et al. (2010), the average speed increase when the size of daphnids increase so the average speed and the average speed class parameter can be nearly correlated to the size increase of the daphnids. Furthermore, some transient decrease can occur during the daphnids molting and may be considered as an "abnormal" change. Some under-detection can be observed in the DaphTox, due to a fast locomotion or clustering of organisms, which hamper the daphnia tracking.

In field conditions, variations of general water quality parameters, such as temperature, turbidity and pH may also affect the behavioral of the animals in BEWSs that could result in the alarm signal. This issue has been addressed in a previous study (Chen et al. 2012). The water quality may vary and toxicity data may more vary especially when chemical toxicity is influenced by water quality (for example metals). False alarms are a major drawback for the use of biomonitors in the field (Puzicha, 1994, Chen et al., 2012).

3.2 Evaluation of "Multi-DaphTrack" system

Our results showed that chemical testing in the "Multi-DaphTrack" system provided sensitive and early responses to chemical stress. In contrast to BEWS systems that can induce false alarms, the "Multi-DaphTrack" system is a reliable test since controls exposed in this system do not provide false positive results. By increasing the number of replicates, a lower variability in behavior was obtained, which highly contributed to enhance the sensitivity of the system. In this study, it is shown that the developed "Multi-DaphTrack" system is very suitable to monitor the average speed over time of exposed-daphnia groups. Thus, behavioral assay in such system may be proposed as a standardized

test for biomonitoring. However, further studies are required to verify if this test meet all criteria required for standardized tests. Finally, this system is able to continuously monitoring behavioral parameters over a period as long as 48 hours. Results obtained in the "Multi-DaphTrack" allow enhancing our understanding in behavioral effects and highlighting the different behavioral profiles induced by chemical exposures over time. Overall, behavioral thresholds obtained from this system may not be as sensitive than chronic toxicity data but still significant effects are detect for sub-lethal concentrations in much less time than Chronic tests.

Tracking 20 daphnia neonates groups simultaneously was technically challenging. A lot of efforts have been done to successfully track Daphnia groups without technical problems and with a reasonable detection rate. The "Multi-DaphTrack" system was successfully optimized, however certain limits may subsist since behavioral tracking technology is not 100% reliable analyses, i.e., artifacts and tracking loss may still occur. Some additional improvements and validations could be also performed on the system. For instance, optical fiber may be used to illuminate the daphnia neonates since fibber transmits the light but isolates the heat from the light source. A light source could be also added to reproduce the day/night photoperiod since the circadian rhythm includes more realistic condition compared to the field (i.e., natural light day/night cycle).

3.3 Positioning on the use of behavioral analysis system in risk assessment

There are advantages and disadvantages to both methods for behavior analysis used in this study. Overall, comparisons of results showed that the DaphToxI® system was less sensitive than the "Multi-DaphTrack" system. On the other hand, in contrast to the "Multi-DaphTrack" system, the DaphToxI® system can be used directly into the field. The "Multi-DaphTrack" system is very sensitive but less representative of field conditions. Exposure results obtained in the "Multi-DaphTrack" system may, hence, be considered, as "model" results but the extrapolation must be performed with caution.

There is an inherent difference between laboratory and field situation. A global interest for the use of BEWS system in biomonitoring campaigns is increasing. In ecotoxicology, the problem of extrapolating results from one scale (i.e., laboratory) to another (i.e., field) is pervasive. The better solution would be to conduct studies at the appropriate scale, but field measurements are difficult to set up and are often costly. BEWS provide a good intermediary between laboratory-performed studies and direct field measurement. When chemical identification is not possible, the use of biomonitors is considered as a good alternative (Yang et al. 2008). Furthermore, instead of focusing on single toxicants, the BEWSs such as DaphToxI® allow detecting behavioral effects induced by mixture of several toxicants present in the field. However, BEWSs are rather suited to monitor acute concentrations (i.e., in case of accidental contamination) than sub-lethal concentrations, which typically occur in the environment.

Ecological risk assessment involves two complementary approaches: it may be performed before toxicant exposure, i.e., **prospective** risk assessment or afterward, i.e., **retrospective** risk assessment (Van den Brink et al. 2011). The goal of **prospective** risk assessment is to assess the likelihood that exposure to a predicted level of toxicant will cause adverse effects on ecosystem. Such assessments are usually performed to meet REACh regulation when chemicals are produced in tonnage or planned to be release in the environment. For the risk assessment of chemicals or the likelihood of vulnerable taxa to be effected, data on exposure (physicochemical analysis) and chemical effects (from QSARs method or measurements) needs to be gathered and then extrapolated to the entire ecosystem. **Retrospective** risk assessment is rather a diagnostic of suspected or monitored impacts and aims to identify the cause of adverse effects that already occurred. Retrospective studies are used as biomonitoring strategy for aquatic environment especially as part of the WFD. This retrospective approach required historical information in order to correlate these measured adverse effects with plausible stressor sources (e.g., which chemical). It is important that prospecting method can be done **at an early stage** to prevent undesirable damage in the ecosystem or heath issue (drinking water). This implies that immediate corrective action should be taken when deleterious effects are detected. Substantial improvements are required to perform efficient risk assessment strategies. Perspectives and evolutions are reported in the technical report on aquatic effect-based monitoring tools (European commission 2014). Physicochemical analysis cannot detect all the various substances present in the environment. Despite specialized analytical techniques such as GC-MS (gas chromatography) and HPLC, which can provide very accurate measurement of chemical compound in water, the price for each analysis is often too costly. For instance, the average cost for an analysis of the 126 US EPA priority pollutants is around 1000 dollars/sample (Bae and Park 2014).

Biomonitoring is typically performed by periodically examining the organisms on the site and compared to historical data. However, this punctual assessment may be inappropriate for detection of pollution peaks since they are typically based of spot sample and not tailored to identify peak concentration, which are most relevant for toxic effects. In contrast, when performed continuously, these methods can **alert at an early stage** to prevent undesirable effects. For instance, the use for detection of pollutions peaks in surface waters may not directly allow the identification of the cause of pollution and the spot of emission, however it may permit to take **immediate action** for e.g. drinking water protection by stopping intake in the water production.

The new **"Multi-DaphTrack" system** can be used in **prospective risk assessment** to characterize behavioral dose-responses of chemicals. Our results showed that test in the "Multi-DaphTrack" system provided very sensitive and early responses to chemical stress. This developed "Multi-DaphTrack" system is very suitable and reliable to understand and evaluate effects of pollutants on the average speed of exposed-daphnia and thus behavioral thresholds (behavioral NOEC/LOEC) may contribute to the evaluation of PNEC and the risk characterization. However,

further studies are required to verify if this test meet all criteria required to be integrated in risk assessment process.

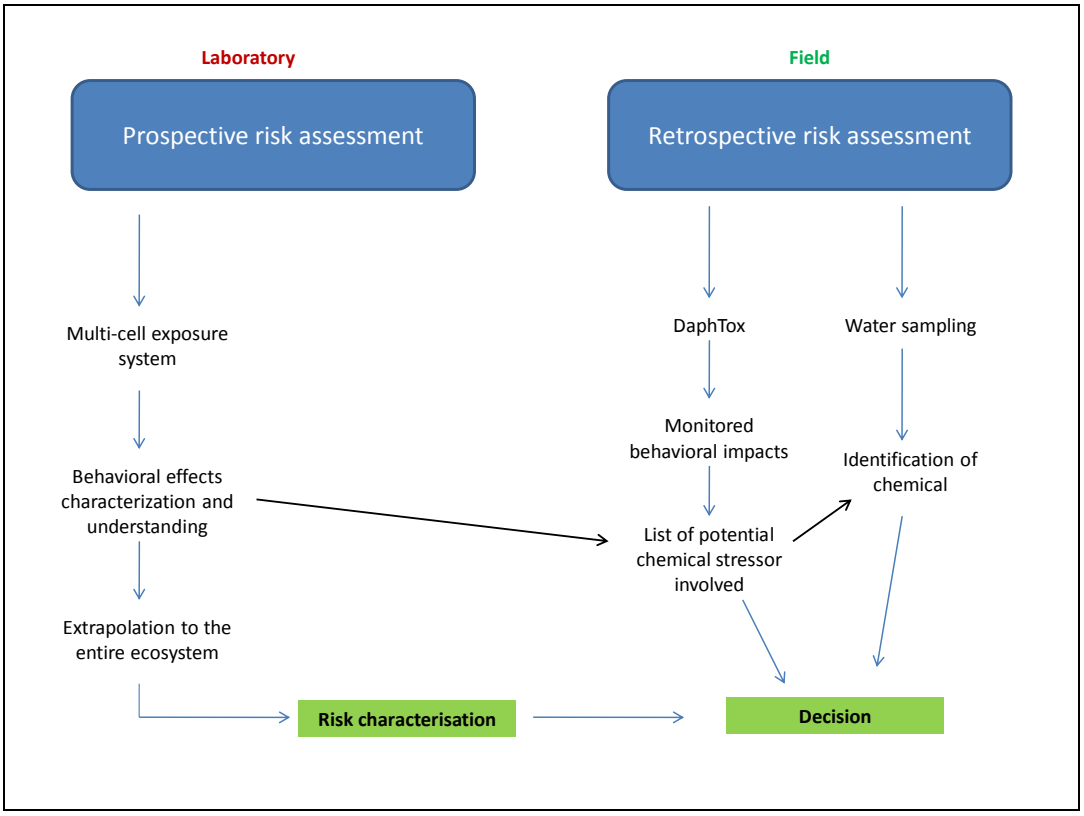


Figure 28: Schematic overview of the possible combination of the "Multi-DaphTrack" system and the DaphTox® system in ecological risk assessment process.

In **retrospective assessment**, biomonitoring is typically performed by periodically water or organisms sampling on the contaminated site to further perform in laboratory exposure or effect assessment and compare results with sites of reference. However, this punctual assessment may prevent to detect pollution since the day of sampling may miss contamination peak. BEWS has the advantage to provide continuous assessment and can detect adverse effects at the moment of actual occurrence. BEWS such the DaphTox are already used in biomonitoring programs. Despite its lower sensitivity compared to the "Multi-DaphTrack" system, the DaphToxI® has been shown to be sensitive to acute contamination and is hence very useful in case of accidental chemical spill. It can be hence proposed as effect-based monitoring tool for **retrospective risk assessment.**

Combination of both systems in risk assessment? It remains unclear how to combine chemical analysis and biological biomarkers. The combination of both "Multi-DaphTrack" system and the DaphTox may provide useful support for pollution biomonitoring. Indeed, prospective risk assessment can be performed with the "Multi-DaphTrack" system and effect results can serve as reference and help in determining the potential causal stressors of retrospective risk assessment performed with the DaphToxI® (Figure 28). The DaphToxI® was initially placed in a mobile station with other BEWS and water sampling system. When an alarm was triggered from one of the BEWS (e.g., the DaphToxI®), water was samples and keep for further chemical analysis. In this way,

behavioral assessment combined with physico-chemical analysis may improve ecological risk assessment. It can also provide a basis for assessing whether industrial and agricultural chemicals are likely to induce adverse effects on ecosystems and hence provide a foundation to take a decision and manage them. Then we can sample water for further chemical analysis and determine compounds, which lead to those effects.

CONCLUSION & PERSPECTIVES

In conclusion, our results showed that the behavior of the freshwater invertebrate cladoceran *Daphnia magna* responds fast and is a sensitive indicator for toxicant stress. We believed that behavioral tests may not replace the actual standard toxicity testing but rather be used to improve the interpretation test results especially for chemicals when their major toxic mode of action act through narcosis or neurotoxicity. The DaphTox® can be used as early warning for acute effect concentrations and "Multi-DaphTrack" system may be used for more in-depth analysis of both acute and chronic effects. Chemical detection and understanding of their effects are valuable information for freshwater management. After a long work of optimization of the exposure conditions together with the enhancement of the detection rate, we finally succeed to obtain a reliable behavior analysis system allowing to monitor up to 20 daphnia groups simultaneously. Behavior analysis using our "Multi-DaphTrack" system could be used as an alternative or complement to the current acute standard test for toxicity assessment of chemicals. With some additional improvements and validations, it also could be used for quality assessment of water bodies and sewages. Linking behavioral responses to the precise mode of action of the chemical remain delicate.

In this study, **the distinction of behavioral profiles** based on the chemical **mode of action** was not straightforward. Our approach was explorative and provides first highlights on behavioral effects of chemicals with different modes of action. However, further analysis should be performed with other chemicals, which involve other modes of action not studied in this study. To obtain a proven correlation, several chemicals with the same mode of action (mode than 2 chemicals) should be compared and a more wide range of chemical mode of action should be investigated (e.g., reactive substances).

In addition, the concentrations of tested chemical **concentrations** must be monitored with **chemical analysis**. Indeed, this study is based on nominal concentrations since chemical concentration analysis could not be performed because of the high chemical analysis cost and technical limitations. To establish dose-responses, nominal concentrations are not enough and the real concentrations of working solutions must be monitored. It is important to follow concentrations to check potential problems during stock solution preparation or dilutions (operator mistakes) and possible degradation of chemical over time. For instance, if the reactive substances are analyzed, it is primordial to follow the degradation kinetic of the substance. Reactive substances are known to degrade very rapidly and are hence the concentration can decrease over time.

In this study, the new developed "Multi-DaphTrack" system has allowed performing several concentrations and replicates but only one single test has been performed for each chemical. Other test should be performed (2 or 3 times) to verify the **repeatability** of the test and possible variations. In

another hand, concentrations tested in the DaphToxI® were too low to induce significant effects. The selection of the **tested concentrations should be refined**: different concentrations with dilution should be measured to obtain a dose-response from behavior effects measured by the DaphToxI®.

Some explanations and hypotheses have been drawn in this study for the understanding of observed behavioral effects. To deal with the understanding of behavioral effects in depth, the observed behavioral effects could be compared and correlated with **effect biomarkers** according to the chemical mode of action. For instance, the cholinesterase activity could be measured and correlated with the increase of the swimming speed induced by acetylcholinesterase inhibitors likewise it was performed in Duquesne and Küster (2010).

Finally, for toxicity assessment of complex mixtures of chemicals present in the environment, complementary behavioral studies on various species from different trophic levels may obviously benefit to risk assessment. Furthermore, the "Multi-DaphTrack" can be utilized for the understanding of behavioral effect induced by mixtures and serve as reference for field measurement with BEWS, e.g. the DaphTox®. In this way, **environmental water sampling** after an induced alarm of BEWS could be analyzed in the "Multi-DaphTrack" system and physico-chemical parameters (organic matter, pH, water hardness) could be measured in parallel. To share similar exposure conditions with the standard acute immobilization test, we decided to exposed organisms with chemicals via direct exposure. In the future, possible **trophic effects** could also be measured in the "Multi-DaphTrack" system by contaminating the food (algae solution) with the toxic.

In this study, tests performed with the DaphTox® was modified in order to compare results with our "Multi-DaphTrack" system. In the literature, other parameters were reported to significantly contribute to alarm induction. Hence, different tests should be performed with the **initial condition of the DaphTox® system**, i.e., feeding, daphnia aged of 24h up to 7 days and water filtration system. We may test known contaminated solution (big volume) which pass by the all complex filtering system to verify that chemicals are not absorbed in the filtration system and induced a decrease of the contaminant concentration exposure.

ANNEX

ANNEX 1: Supporting information article 1- Chapter II

(a) Results from the standard immobilisation acute toxicity test

Tested concentrations were selected based on the EC_{50} (48H) value of 0.9 µg/L (Lewandowska 2004) and modelled to reach 0 up to 100% of immobilisation with a separation factor between concentrations dilution factor of 2.2. For each replicate (four replicates per condition), five neonates were incubated at 20± 2°C in darkness in 10 mL of control (ISO water) or tested solution at one of the seven concentrations tested: 0, 0.19, 0.43, 0.94, 2.1, 4.5 and 10 µg/L of esfenvalerate. During the experiment, no food was added. Four solvent controls with 0.01% of MeOH were also performed to verify the lack of toxicity of methanol. The 24 and 48 hours EC_{50} for *Daphnia magna* exposed to esfenvalerate were calculated to be respectively 1.0 µg/L and 0.89 µg/L. No immobility was observed in controls with and without 0.01% MeOH.

(b) Configuration of the test condition on the Bbe® Daphnia toximeter

For daphnids acclimation, an ISO water solution circulated with an optimal flow-through of 33 mL/min at 20 ± 1 °C. After 2 hours, exposure was started by replacing the ISO water with the test solution. This latter solution was placed in closed circuit after complete ISO water evacuation (time previously estimated at 3 minutes by colorimetric measurement). To be consistent with the immobilisation test, the experiments were conducted without food supply in contrast to recommended test protocols for this system.

ANNEX 2: Supporting information article 2- Chapter III

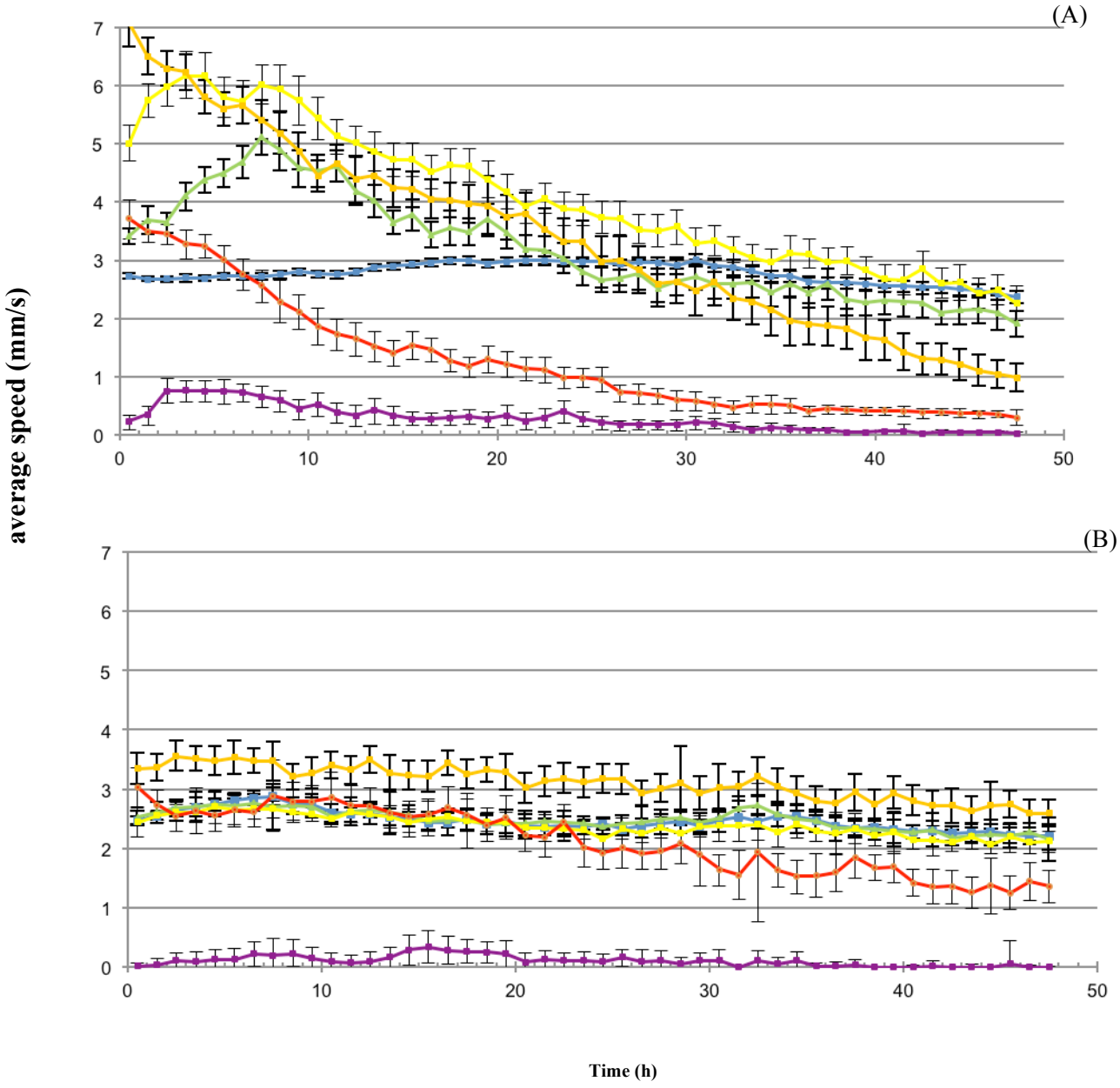
(a) Chemical tested

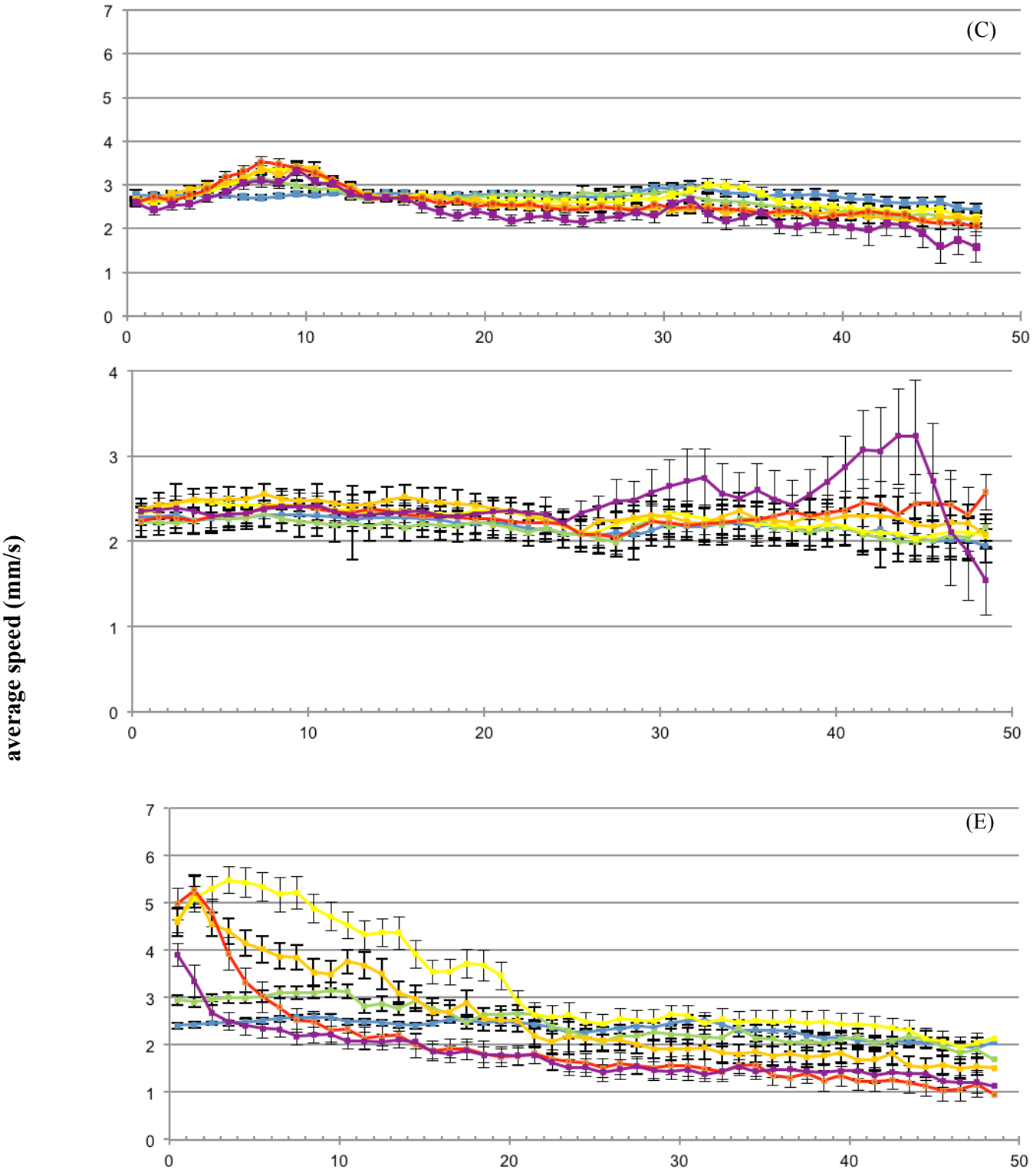
All chemicals products were purchased at VWR®, France. Esfenvalerate (CAS: 66230-04-4), caffeine (CAS: 58-08-2), fipronil (CAS: 120068-37-3), cypermethrin (CAS: 52315-07-8), imidacloprid (CAS: 138261-41-3), sertraline (CAS: 79617-96-2) and copper sulfate pentahydrate (CAS: 7758-99-8) were provided by as solid suspension of 250 g (purity 99%) and of 25 mg for trichlorfon (CAS: 52-68-6) (purity 99%). Carbofuran (CAS: 1563-66-2) and abamectin (CAS: 71751-41-2) were supplied as solution in pure methanol (1mL of 1g/L and 1mL of 100µg/mL respectively, purity 99%). Ethanol (CAS: 64-17-5), isopropanol (CAS: 67-63-0) were supplied as pure solution (purity 99%). All tested solutions were prepared with artificial reconstituted freshwater (ISO 6341 2012). Since esfenvalerate, carbofuran and abamectin have a low solubility in water, concentrated solution were prepared in methanol to help solubilisation in water as it is recommended by OECD guidance document for difficult substances (OECD 2000) and was then diluted with ISO to obtain the percentage not exceeding 0.01% in all treatments including control solvent.

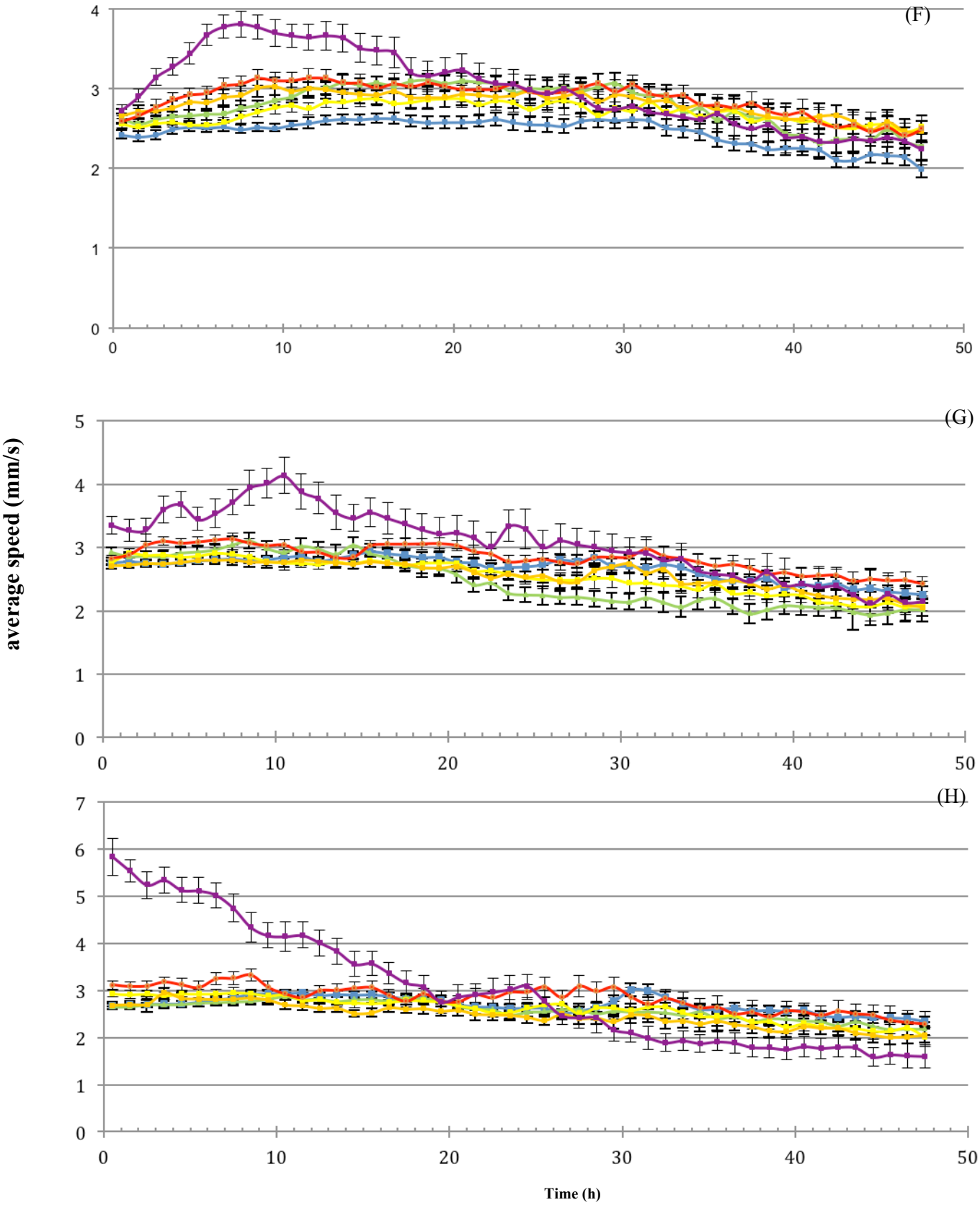
(b) Configuration of the test condition on the Bbe® Daphnia toximeter

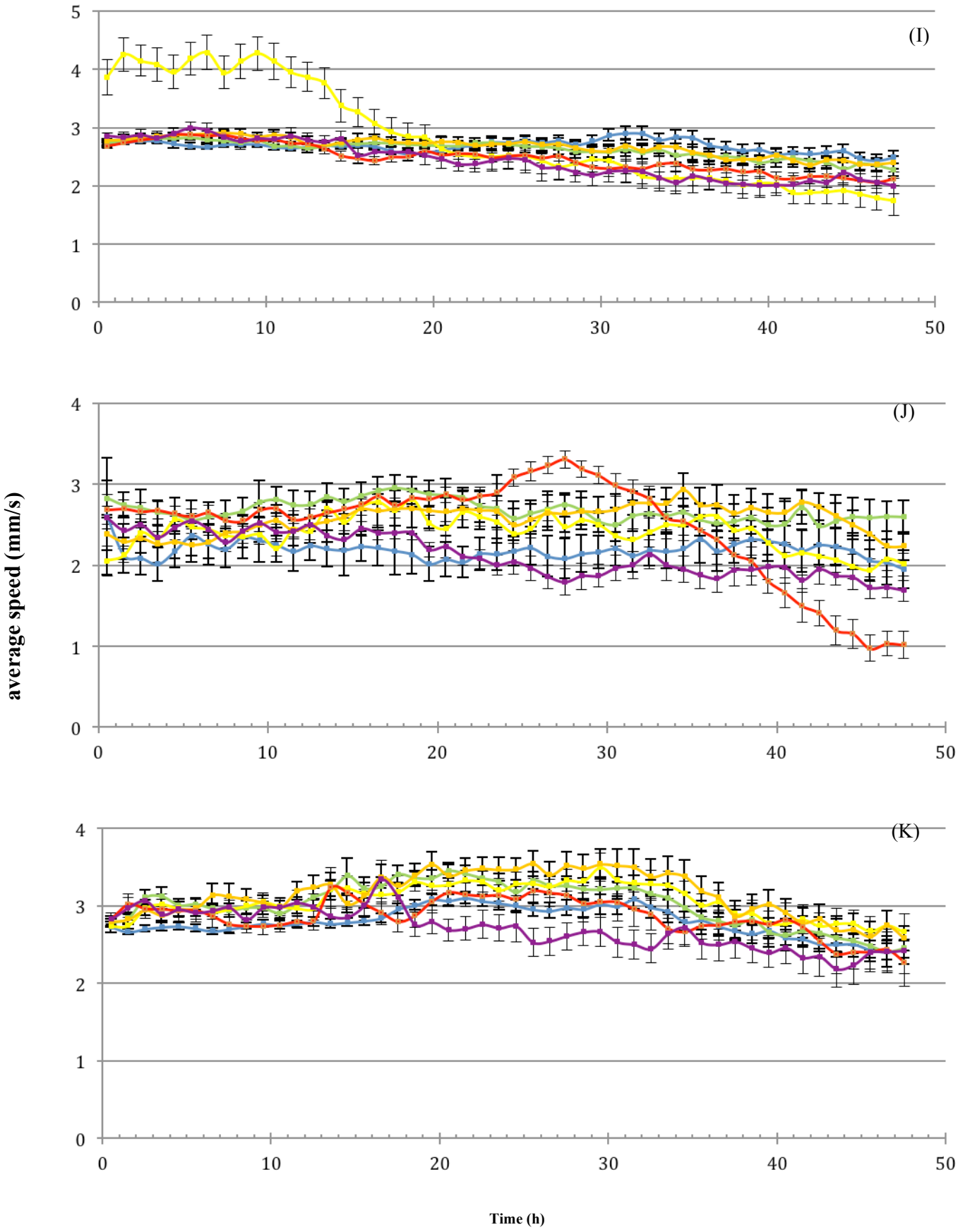
For daphnids acclimation, an ISO water solution circulated with an optimal flow-through of 33 mL/min at 20 ± 1 °C. After 2 hours, exposure was started by replacing the ISO water with the test solution. This latter solution was placed in closed circuit after complete ISO water evacuation (time previously estimated at 3 minutes by colorimetric measurement). To be consistent with the immobilization test, the experiments were conducted without food supply in contrast to recommended test protocols for this system.

(c) Average swimming speed ± standard error (per hour) of *Daphnia magna* neonates exposed to several concentrations of (A) isopropanol (B) ethanol (C) copper sulfate (D) trichlorfon (E) esfenvalerate (F) fipronil (G) sertraline (H) cypermethrin (I) caffeine (J) abamectin (K) carbofuran and (L) imidacloprid for 48 hours in the "Multi-DaphTrack" system.




(C)
(E)
average speed (mm/s)


(F)
(G)
(H)
average speed (mm/s)
Time (h)


(I)
(J)
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average speed (mm/s)
Time (h)
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4
5
0
10
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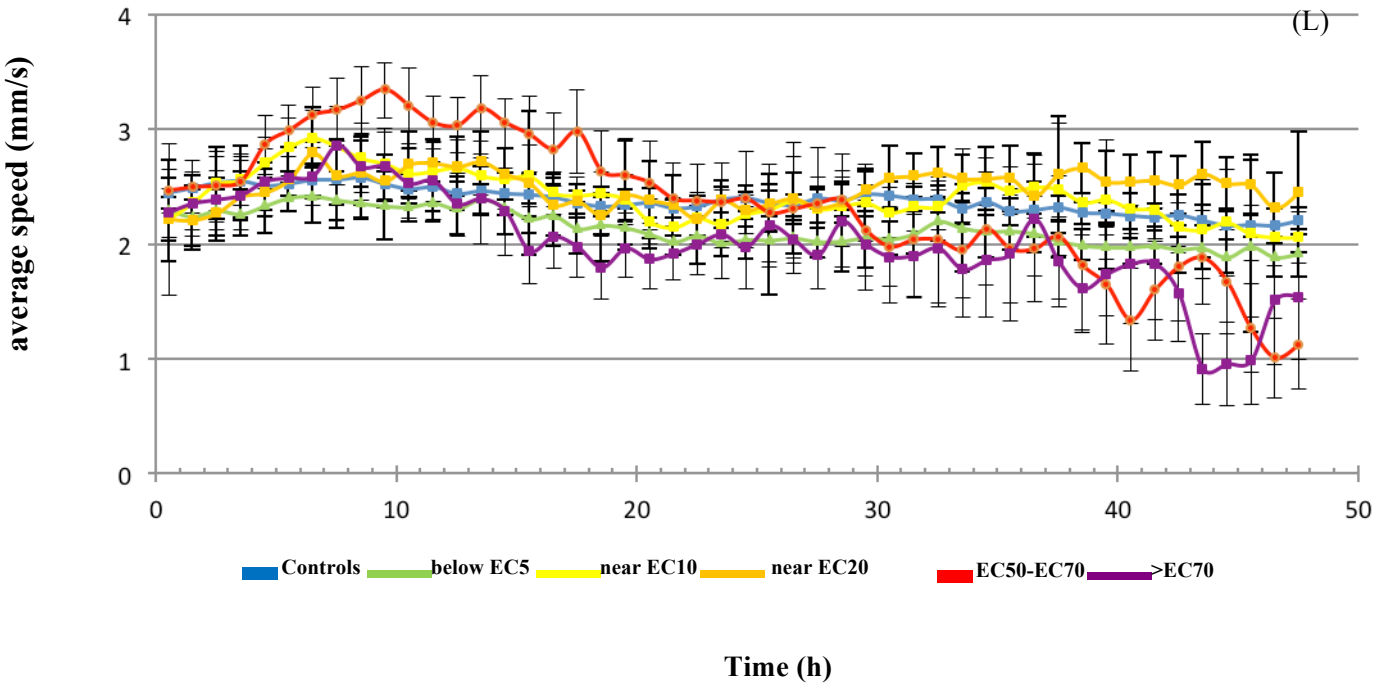
30
40
50

(L)


average speed (mm/s)
4
3
2
1
0
0
10
20
30
40
50
Controls
below EC5
near EC10
near EC20
EC50-EC70
>EC70
Time (h)

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